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Assessment of recent developmental immunotoxicity studies with bisphenol A in the context of the 2015 EFSA t-TDI

Ellen V.S. Hessel¹, Janine Ezendam¹, Fleur A. van Broekhuizen², Betty Hakkert², Jamie DeWitt³, Berit Granum⁴, Laurence Guzylack⁵, B. Paige Lawrence⁶, Andre Penninks⁷, Andrew A. Rooney⁸, Aldert H. Piersma^{1,9}, Henk van Loveren^{1,10}

¹ Center for Health Protection and, ² Center for Safety of Substances and Products, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands,

³ Department of Pharmacology and Toxicology, East Carolina University, Greenville, North Carolina, USA

⁴ Department of Toxicology and Risk Assessment, Norwegian Institute of Public Health (NIPH), Oslo, Norway.

⁵ Department of Intestinal Development, Xenobiotics, and Immunotoxicology, Institut National de la Recherche Agronomique (INRA), Research Centre in Food Toxicology (Toxalim), Université de Toulouse, INRA, Toulouse, France.

⁶ Department of Environmental Medicine and Department of Microbiology and Immunology, University of Rochester School of Medicine & Dentistry, Rochester, New York, USA

⁷ Formerly Department of Toxicology and Risk Assessment, TNO Triskelion BV, Zeist, The Netherlands

⁸ National Toxicology Program, Office of Health Assessment and Translation, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA,

⁹ Institute for Risk Assessment Sciences, Veterinary Faculty, University of Utrecht, Utrecht, The Netherlands

¹⁰ Currently Department of Toxicogenomics, Maastricht University, Maastricht, The Netherlands

Summary/Abstract

Humans are exposed to bisphenol A (BPA) mainly through the diet, air, dust, skin contact and water. There are concerns about adverse health effects in humans due to exposure to bisphenol A (BPA). The European Food Safety Authority (EFSA) has extensively reviewed the available literature to establish a temporary Tolerable Daily Intake (t-TDI). This exposure level was based on all available literature published before the end of 2012. Since then, new experimental animal studies have emerged, including those that identified effects of BPA on the immune system after developmental exposure. These studies indicate that developmental immunotoxicity might occur at lower dose levels than previously observed and on which the current EFSA t-TDI is based. The Dutch National Institute for Public Health and the Environment (RIVM) organized an expert workshop in September 2015 to consider recently published studies on the developmental immunotoxicity of bisphenol A (BPA). Key studies were discussed in the context of other experimental studies. The workshop concluded that these new experimental studies provide credible evidence for adverse immune effects after developmental exposure to BPA at 5 µg/kg BW/day from gestation day 15 to postnatal day 21. Supportive evidence for adverse immune effects in similar dose ranges was obtained from other publications that were discussed during the workshop. The dose level associated with adverse immune effects is considerably lower than the dose used by EFSA for deriving the t-TDI. The workshop unanimously concluded that the current EFSA t-TDI warrants reconsideration in the context of all currently available data.

Introduction

In 2015, EFSA published its Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs (1). The EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) endorsed the toxicity section of the opinion in December 2013. In this opinion, the risk characterization is based on peer reviewed literature published before the end of 2012 (1). The EFSA opinion (1) describes the assessment of the risks to public health associated with BPA exposure. We quote: "BPA toxicity was evaluated by a weight of evidence approach. "Likely" adverse effects in animals on kidney and mammary gland underwent benchmark dose (BMDL10) response modelling. A BMDL10 of 8960 µg/kg bw per day was calculated for changes in the mean relative kidney weight in a two generation toxicity study in mice (2). No BMDL10 could be calculated for mammary gland effects. Using data on toxicokinetics, this BMDL10 was converted to a Human Equivalent Dose (HED) of 609 µg/kg bw per day. The CEF Panel applied a total uncertainty factor of 150 (for inter- and intra-species differences and uncertainty in mammary gland, reproductive, neurobehavioural, immune and metabolic system effects) to establish a temporary Tolerable Daily Intake (t-TDI) of 4 µg/kg bw per day. By comparing this t-TDI with the exposure estimates, the CEF Panel concluded that there is no health concern for any age group from dietary exposure and low health concern from aggregated exposure. The CEF Panel noted considerable uncertainty in the exposure estimates for non-dietary sources, whilst the uncertainty around dietary estimates was relatively low." (1) Based on the current t-TDI derived by EFSA, exposure estimates from non-dietary sources such as medical devices or thermal paper were evaluated by the EU Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) or the Risk Assessment Committee (RAC), respectively. SCENIHR (2015) concluded that a risk of adverse effects from BPA exposure may exist for neonates in intensive care units, young children undergoing prolonged medical treatment and dialysis patients when the BPA is directly available for systemic exposure after non-oral exposure routes. Additionally, the benefits to be derived from the use of these medical devices must also be considered (3). RAC published its opinion on the human health hazards presented by BPA in the context of a restriction proposal for the use of BPA in thermal paper under REACH. For consumers, the RAC concluded that the risks from BPA exposure via thermal paper receipts are adequately controlled. For cashiers, the RAC concluded that the risks of exposure via thermal paper receipts are not adequately controlled. These risks include potentially severe effects on the unborn children of pregnant female workers (4).

Among the issues leading to their uncertainty factor of 150, EFSA specifically mentions immune effects (1). EFSA states that: "there are indications that BPA may be linked to immunological outcomes in humans, although in view of the limitations of the studies only limited conclusions can be reached and it cannot be ruled out that the results are confounded by diet or concurrent exposure factors. The associations do not provide sufficient evidence to infer a causal link between BPA exposure during pregnancy or in childhood and immune effects in humans." As to animal studies published before the end of 2012, EFSA notes that: "whereas the studies lend support to the notion that immunological effects may be elicited by BPA, all these studies suffered from shortcomings in experimental design and reporting. Therefore, a dose-response cannot be confidently established. It is currently not clear whether immunotoxicity is an endpoint of concern for BPA. The CEF Panel noted that this type of effect is insufficiently covered by current testing guidelines, and potential immunotoxicity therefore currently presents an uncertainty area in BPA risk assessment, deserving further consideration."

More recently, additional studies on immune effects following BPA exposure have emerged in the scientific literature. These recent publications include both epidemiological studies and experimental studies using animal models. Although the epidemiological studies published after 2012 (5-8) provide additional evidence of a potential association between BPA and immune effects, the available human studies corroborated EFSA's conclusions as described above, indicating that a causal link cannot be

identified from human studies due to limitations. For example, spot urinary BPA levels do not provide a reliable measure for exposure over time. Moreover, the possible and unknown time lags between relevant exposure windows and the age at observed effects complicate the interpretation of associations. Observational and cross-sectional studies by definition provide evidence for associations, but cannot prove cause and effect. This assessment is in line with a recently published review on this subject (9).

In contrast, well-designed animal studies can provide information regarding cause and effect relationships. With this in mind, more recent animal studies are noteworthy because their findings might drive reconsideration of the t-TDI determined by EFSA. The Dutch National Institute for Public Health and the Environment (RIVM) therefore gathered a group of international experts knowledgeable in immunotoxicology, regulatory toxicology and chemical risk assessment at a workshop assessing these recent studies, which was held in the Netherlands on 29 September 2015. The workshop participants unanimously concluded that reconsideration of the 2015 EFSA t-TDI is warranted, as adverse effects on the immune system were observed in new animal studies, with the lowest effective dose considerably below the effective dose used to calculate the Human Equivalent Dose (HED) on which EFSA based the derivation of their t-TDI.

Workshop scope and outline

After the EFSA evaluation, peer reviewed research publications emerged that reported effects on the immune system after BPA exposure during development. During the workshop, the participants considered that the two key studies performed by Ménard and colleagues needed further evaluation based on the identified immune effects at low BPA dosages (10, 11). At the workshop, these experimental studies were presented in detail by one of the authors Dr. Laurence Guzylack from INRA (Institut National de la Recherche Agronomique). Another study that was considered by EFSA was a report by Bauer and colleagues in 2012, which was presented by Dr. Paige Lawrence from the University of Rochester (12). In this study, immune effects after developmental exposure to a range of low dosages of BPA were studied. The participants evaluated the design and results of the different experimental studies performed in these three publications in detail. The outcomes were put in the context of other available animal studies that identified immunotoxic effects of BPA (13-18). The workshop considerations will be represented in this report.

Detailed description of the Ménard studies

Two manuscripts by Ménard et al. (2014) (10, 11), that were published after the closure date of the literature search for the 2015 EFSA opinion, reported on immune effects of pre- and postnatal developmental low dose exposure to BPA on oral tolerance and immunization to OVA (10, 11) and on host resistance to infection with an intestinal parasite (11) in rats. Oral tolerance induction is a tightly regulated immunological process that takes place in the gut-associated lymphoid tissue. It is characterized by an absence of cellular and humoral immune responses to an orally administered food allergen. A disturbance of this process can result in food allergy. Host resistance models are used to assess if the immune system is capable of mounting an immune response to a pathogen. Impaired host resistance is demonstrated by an increased susceptibility to the pathogen.

Effects of BPA on oral tolerance and immunization were investigated in two studies using different experimental designs, which are summarized in detail in Tables 1 and 2 (10). The aim of the study 1 was to assess effects of three dose levels of BPA (0.5, 5 or 50 µg /kg BW/day) on the systemic immune response to OVA in tolerized and immunized Wistar rats (Table 1: study 1). Rats were treated orally with BPA from their 15th day of gestation to postnatal day 21. At the age of 45 days, offspring rats were tolerized by a single oral gavage with 20 mg of OVA. The OVA-immunized rats were

gavaged with the vehicle only. Systemic immunization was established by priming rats subcutaneously (s.c.) twice with OVA in Complete Freund Adjuvant (CFA). Using this protocol, it was shown that rats not exposed to BPA were tolerized to OVA, because OVA-specific IgG titers were 50 times lower in tolerized control rats compared to OVA-immunized control rats. Perinatal BPA exposure impaired the induction of oral tolerance as evidenced by increased OVA-IgG titers. Already at 0.5 µg/kg BW/day a significant increase in average IgG titer was found. The highest average titers were observed in the mid dose group at 5 µg/kg BW/day. In OVA-immunized rats, average titers were enhanced as well by perinatal BPA exposure at 5 and 50 µg/kg BW/day.

To further study the effects of BPA on oral tolerance and immunization, a second experiment (Table 2, study 2) was performed (10). Rats were treated orally with single daily doses of 5 µg/kg/day BPA from the 15th day of gestation to postnatal day 21. This dose was selected based on the first study, showing that the 5 µg/kg BW/day had the most pronounced immune effects. In study 2, different immune parameters were measured after multiple oral OVA challenges both in tolerized and immunized offspring rats. The challenge protocol was different from the first study, because the protocol used in study 2 allowed the assessment of the local immune responses in the colon as well as the systemic immune response to OVA. Systemic immune effects of BPA were assessed in spleen cells. It was shown that BPA at 5 µg /kg BW/day increased the number of activated T cells in the spleen, but did not have an effect on regulatory (CD4⁺CD25⁺FoxP3⁺) T cells. BPA increased cell proliferation and IFN γ production by spleen cells *ex vivo* restimulated with OVA as well. In OVA-immunized rats, an increase of IFN γ production by spleen cells was observed, but cell proliferation and IL-10 secretion were not affected by BPA exposure. BPA had an effect on the local immune response in the colon as well. It was shown that in OVA-tolerized rats, that upon BPA exposure neutrophil infiltration and IL-10 and IFN γ levels in the colon were increased.

In a third study of Ménard et al. (10, 11) effects of BPA were studied using the same protocol for OVA tolerization and immunization as in study 1 (10). However, the age at which oral tolerance was induced in this study 3 was at 25 days (juvenile age) (Study 3, Table 3) (11) and not at 45 days (10). Oral tolerance was induced by this protocol as evidenced by a 50-fold lower anti-OVA IgG titer in OVA-tolerized control rats compared to OVA-immunized control rats. In contrast to the previous studies, perinatal BPA exposure of dams did not have any effect on the OVA-specific IgG titers in offspring rats tolerized and immunized to OVA (11). Hence, oral tolerance induction was not impaired when rats were exposed to BPA at a juvenile age. The cellular response, however, was affected by juvenile BPA exposure; as evidenced by decreased OVA-induced IFN γ production by spleen cells both in OVA immunized and tolerized rats. These results are in line with the observed decreases of Th cells, regulatory (CD4⁺CD25⁺FoxP3⁺) T cells and dendritic cells in the spleen and mesenteric lymph nodes in BPA exposed tolerized rats (11).

Ménard et al. (2014) investigated the effects of perinatal BPA exposure (5 µg /kg BW/day) from the 15th day of gestation to postnatal day 21 in rats on host resistance (Study 4, Table 5) (11). Offspring rats were infected with the nematode *Nippostrongylus brasiliensis* (*N. brasiliensis*) at day 25 of age (11). One week later rats were euthanized. Perinatal exposure to BPA impaired host resistance, demonstrated by increased fecal larvae number compared to infected controls not exposed to BPA. No effects on total IgE levels were found, but decreased neutrophil infiltration was observed after BPA exposure. Furthermore, BPA increased Th2 (IL13, IL4), anti-inflammatory (IL10) and pro-inflammatory (GRO, IFN γ) cytokines compared to infected rats not exposed to BPA. Nematode infections normally decrease Th1 responses and trigger Th2 responses that are required to eliminate the nematode. The increase of IFN γ may explain the impaired clearance of the parasite (11). Results of the Ménard studies are summarized in Table 4.

Detailed description of the Bauer study

In the study of Bauer et al. (2012) (12), effects of pre- and postnatal developmental BPA exposure were investigated in two established mouse models for respiratory allergy (12) (experimental designs are described in Table 5). Pregnant C57BL/6 dams were gavaged with 0, 0.5, 5, 50, or 500 µg BPA/kg BW/day from gestational day 6 until postnatal day 21. In both allergy models, ovalbumin (OVA) was used as an allergen, but the route of antigen administration during the sensitization phase differed. In the mucosal sensitization model, sensitization was induced by intratracheal exposure to OVA alone or to OVA together with a low dose of lipopolysaccharide (LPS) as an adjuvant. In the systemic sensitization model, sensitization was induced via intraperitoneal injection of OVA together with alum as an adjuvant. Elicitation responses were evoked by airway challenge to OVA aerosols using the same protocol for both sensitization protocols. The intraperitoneal model (systemic sensitization model) is more widely employed, but uses a route of exposure that is not relevant to humans. On the other hand, the mucosal model uses the physiological route of exposure in humans. It has been recognized that both the route of exposure for sensitization as well as the adjuvants will differentially influence the immune response and underlying mechanisms (19, 20). Although both models can be used to assess the impact of BPA on the immune system, the mucosal model is generally considered to be more relevant to human health.

In the mucosal sensitization model, immune effects of pre- and postnatal developmental exposure to BPA (50 and 500 µg/kg BW/day) were sex-specific. In the female offspring exposed to 500 µg/kg BW/day BPA, lymphocyte infiltration in the lungs was significantly enhanced in OVA-sensitized mice compared to OVA-sensitized vehicle controls. Histopathology findings showed a dose-dependent increase of airway inflammation in female offspring exposed to 50 and 500 µg/kg BW/day. The findings in male offspring were less pronounced and inconsistent and the overall lung inflammation was not different among the groups of males. Airway hyperreactivity was not measured, consequently there is no information as to whether these effects will impair airway function. Yet, in this model, effects of lower levels of BPA were studied as well (0.5 and 5 µg/kg BW/day), but these dose levels did not affect immunological metrics of allergic responses in the lung (12).

In contrast, in the systemic sensitization model, pre- and postnatal developmental BPA exposure resulted in dampening rather than enhancement of the allergic response in the airways. These effects were observed in mice exposed to 0.5, 5 or 50 µg/kg BW/day. In these dose groups, the influx of eosinophils into the airways was lower compared to female offspring from dams in the vehicle control group. Eosinophil influx was not decreased in mice exposed to 500 µg/kg BW/day. No effects on lymphocyte or neutrophil infiltrates were found. Suppression of the allergic response to OVA was supported by a trend toward fewer regulatory (CD4⁺CD25⁺FoxP3⁺) T cells in the lung. Furthermore, in all groups exposed to BPA, including the 500 µg BPA group, OVA-specific IgE levels were reduced, showing that the systemic immune response was affected by BPA as well. Airway responsiveness, as a measure for functioning of the airways, was not affected in offspring exposed to BPA; however, this was only measured in offspring of dams dosed at ≥50 µg/kg BW / day (12).

Workshop discussion and conclusions of the key studies

The studies discussed in detail during the workshop demonstrate that perinatal exposure to doses of BPA lower than those used to establish the t-TDI set by EFSA have an adverse effect on the immune system. These effects are clearly dependent on the experimental model used, the period of exposure to BPA and the age at which the immune system is challenged with an allergen or pathogen. The study by Ménard et al. (10) was considered as the key study, since it measured dose-related effects of BPA starting from 0.5 µg/kg BW/day. An important aspect that was discussed during the workshop was the robustness of the model and this was done by evaluating the experimental design, the plausibility of the related endpoints, and the consistency in the results across studies and across

endpoints to support the observed effect. The workshop participants agreed that there is as yet no scientific consensus on worldwide standards for experimental immune oral tolerance models. However, the same holds true for many other experimental immune models. Such models have not been validated or standardized in great detail. Moreover, test guidelines for a broad spectrum of immune effects are lacking. The experimental designs of the studies performed by Ménard et al. (10) were evaluated thoroughly by the workshop participants and there was consensus that these were sound. In addition, the immune effects measured in study 2, in which only the mid dose of 5 µg/kg BW/day was tested, were consistent with the observed effects on the OVA-IgG titers observed in first study in the same publication, and strengthened confidence in these findings (10). A significant increase of OVA-IgG titers was observed at the 0.5 µg/kg BW/day as well, but because this effect was the only parameter studied at this dose level there was no consensus amongst the participants of the workshop on the relevance of these findings in terms of adversity. Some participants were convinced that the effect on OVA-specific IgG at this dose should be considered adverse, since it is the most important read-out in this model, and that a broader range of endpoints affected at the higher dose supported it. Others had some reservations on the interpretation of this effect, given the absence of assessment of other parameters at this dose level to support the effect on OVA-IgG titers. Therefore, it was concluded that the results obtained at 5 µg/kg BW/day are considered relevant for the risk assessment for BPA. The observations that at this exposure level BPA impaired host resistance as well, as shown by an increased susceptibility to intestinal parasitic infections, strengthens this conclusion (11).

In the study of Bauer et al. (2012), pre- and postnatal developmental exposure to 50 µg/kg BW/day enhanced lung inflammation in the mucosal sensitization model (12). In mice exposed to lower BPA doses (0.5 and 5 µg/kg BW/day), the immune response was not significantly affected. Hence, in the mucosal sensitization model, effects on the immune response to OVA sensitization and challenge were observed at a higher dose than in Ménard et al. (10). Interestingly, in the systemic sensitization model described by Bauer et al. (12), pre- and postnatal developmental exposure to 0.5 µg/kg BW/day had an effect on the immune system. At this dose, BPA decreased rather than increased the immune response to OVA, as was observed in the mucosal sensitization model. Possible mechanisms that can explain the differential effects of BPA found in these two sensitization models were not discussed at the workshop. Nevertheless, the workshop participants concluded that although the directionality of the response differed compared to the mucosal sensitization model, the results suggest that BPA may interfere with the function of immune system even at a dose of 0.5 µg/kg BW/day. This provides additional experimental evidence on the effects at these BPA exposure levels.

Summary of critical findings in the key studies

The studies presented during the workshop that investigated the effects of low dose exposure to BPA are summarized in short below to provide an overview of immune effects observed at these low doses.

- Mucosal sensitization model (12): BPA enhanced the immune response at **50 µg/kg BW/day**.
- Systemic sensitization model (12): BPA reduced the immune response at **0.5 µg/kg BW/day**.
- Oral tolerance model (study 1) (10): BPA impaired oral tolerance induction at **0.5 µg/kg BW/day** in rats tolerized at day 45.
- Oral tolerance model (study 2) (10): BPA impaired oral tolerance induction at **5 µg µg/kg BW/day** in rats tolerized at day 45. At this dose, BPA increased both systemic cellular responses and local mucosal immune responses in the colon.
- Oral tolerance model (study 3) (11): BPA suppressed cellular immune responses at **5 µg/kg BW/day**, but did not affect oral tolerance induction at this dose in rats tolerized at day 25.
- Host resistance model (study 4) (11): BPA increased susceptibility to the intestinal nematode infection at **5 µg/kg BW/day**.

Additional Animal studies evaluated

In addition to the studies of Ménard et al. (10, 11) and Bauer et al. (12), the workshop participants identified three other reports published after 2012 and in which immune effects of BPA were studied in different experimental immune models (13, 15, 16). Additionally, three other studies (14, 17, 18) already evaluated by EFSA were reconsidered due to their relevance to the new studies. Although the experimental design of these studies was not discussed in detail at the workshop, these additional studies are described as supportive evidence for the effects of BPA on the developing immune system identified in the key studies as described above. The BPA dosages used were higher than the dosages in the three key studies; moreover, when evaluated previously, EFSA considered them as insufficient to confirm effects of BPA on the immune system (14, 17, 18). In the following sections, key findings are summarized and integrated with the findings of the Ménard and Bauer reports. Table 5 summarizes the experimental design of these studies, and the main findings of these studies are presented in Table 6.

Oral Immunization and Oral Tolerance models

In addition to the Ménard et al. studies (10,11), one other study investigated the effects of BPA using oral immunization and oral tolerance models (13). These studies differed with regard to the mouse strain, timing, dose, and route of exposure to BPA, the model food allergen used, the sensitization and challenge protocol performed, and the endpoints measured (Table 5). In the Nygaard et al., 2015 study (13), C3H/HeJ mice were exposed to BPA in their drinking water (0, 1, 10 or 100 µg/ml (lowest dose 180-340 µg/kg BW/day, see Table 5 for all corresponding calculated BPA exposure levels in this study in µg/kg BW/day) from mating until the end of the experiment. Effects of BPA exposure were studied in a food allergy model and an oral tolerance model using lupin as an allergen. Oral tolerance to lupin was successfully induced by a single oral gavage of 5 mg in 3-week-old offspring, as evidenced by lower anaphylaxis scores compared to the food allergic control mice. Using this anaphylaxis score, developmental BPA exposure did not have any effects on oral tolerance induction. However, pre- and postnatal developmental exposure to BPA at 100 µg/ml increased cytokine levels (IL13, IFN γ and IL2) in splenocytes from the food allergic mice, and decreased mouse mast cell protease (MMCP)-1 serum levels in mice immunized and tolerized to lupin (13).

Unlike the Ménard studies, BPA did not affect oral tolerance to lupin in this mouse food allergy model. This may be explained by the use of a different food allergy model, animal species, route of exposure to BPA and/or the specific metrics of immune function used in these studies.

Host resistance model

Roy et al., 2012 (14) studied effects of BPA exposure on host resistance in mice infected with influenza A virus. Female C57BL/6 mice were exposed to BPA (0 or 50 µg/kg BW/day) by oral gavage from gestation day 6 to weaning at postnatal day 21. Developmental exposure to BPA neither compromised disease-specific adaptive immunity against the virus infection, nor reduced the host's ability to clear the virus from the infected lung. However, maternal exposure to BPA transiently reduced the extent of infection-associated pulmonary inflammation, as shown by a decrease of the pro-inflammatory cytokine TNF- α , the chemokines RANTES (CCL5), IP-10 (CXCL10)) and the anti-viral gene expression in lung tissue (14).

BPA had a slight impact on the pulmonary inflammatory and anti-viral response to influenza A virus. However, this effect did not impair the host resistance since mice were capable of clearing this influenza virus. The study of Ménard et al. (11) provides evidence that BPA can impair host resistance to intestinal nematode infections in rats. It is inappropriate to compare these host resistance models, since they studied categorically different pathogens in distinct anatomical sites of two different host organisms, and the clearance of viruses and nematodes involves different immunological mechanisms.

Respiratory allergy model

Among all studies discussed by the workshop participants, studies using the respiratory allergy model were common (Table 5) (12, 13, 15-18). Differences in sensitization and challenge in the protocols were observed, as well as in the route and dosing of BPA, the exposure period, and measured endpoints. Despite these differences, some commonalities were noted.

In the Petzold et al., 2014 (15) study, female BALB/c mice were exposed to BPA (0 or 5 µg/ml, or roughly 450 µg/kg BW/day) via their drinking water one week before mating, though exposure was discontinued during the mating period of 1 week. Afterwards, BPA was given to pregnant mice either until parturition (prenatal exposure) or until weaning, when pups were 3 weeks old (perinatal exposure). To induce an allergic asthma like phenotype, mice were sensitized to OVA together with alum as an adjuvant (i.p.), followed by an intranasal OVA challenge, and airway hyperreactivity assessment with methacholine. BPA exposure during gestation and via lactation had no significant effect on airway hyperreactivity or other measured endpoints in the offspring compared to controls. In contrast, postnatal exposure from birth until the last OVA challenge increased eosinophilic inflammation in the lung, induced airway hyper reactivity and antigen-specific serum IgE levels in OVA-sensitized adult mice compared to mice without BPA exposure (15).

In the O'Brien et al., 2014 (16) study, BALB/c mice were exposed to BPA in their diet throughout gestation and lactation until postnatal day 21 (50 ng, 50 µg, or 50 mg BPA/kg of rodent chow = 7.5 ng, 7.5 µg, or 7.5 mg BPA/kg BW/day). Respiratory inflammation was induced by sensitizing offspring at 12 weeks of age to OVA together with the adjuvant Alum (i.p.). Mice were subsequently challenged with aerosolized OVA. Serum anti-OVA IgE levels were increased 2-fold in offspring exposed to 50 µg and 50 mg BPA/kg diet, compared with control animals (in females also in the 50 ng/kg diet group). In addition, OVA-stimulated splenocytes showed IL-13 increases in the 50 µg/kg diet and the 50 mg/kg diet groups, whereas IFN γ increased in all dosages (16).

In the Nygaard et al., (2015) (13) study, BALB/c mice were exposed to BPA (0; 10 or 100 µg/ml study, (corresponding calculated BPA in this study in µg/kg BW/day see Table 5) in their drinking water from mating until start of the lactation period. Mice were sensitized by OVA i.p. without adjuvant and challenged with 10 mg OVA intranasally (i.n.). The number of eosinophils in the bronchoalveolar lavage (BAL) fluid was increased in the 100 µg/ml BPA group compared to the control group (13).

Nakajima et al. (2012) (17) exposed BALB/c mice to BPA in their drinking water at 0 or 10 µg/ml (around 900 µg/kg BW/day, Table 5). Using cross-fostering, pups were divided into three groups based on exposure period to BPA: Group 1: exposed from 1 week before mating to PND25; Group 2: exposed postnatally (PND2 to PND25); Group 3: exposed from 1 week pre-mating to PND2. Respiratory allergy was induced in half of the pups from each exposure group by sensitizing them (i.p.) with low levels of OVA together with alum; the remaining mice were exposed to the vehicle (PBS). All pups (sensitized or not) were challenged with aerosolized OVA. Pups exposed to BPA in utero and through breast milk (group 1), or in utero only (group 3), displayed an enhanced sensitivity to the asthma phenotype as evidenced by increased airway hyperreactivity to methacholine compared to control mice or to pups only exposed to BPA postnatally (group 2). This was accompanied by an increase in the number of eosinophils in the bronchoalveolar lavage (BAL) fluid in groups 1 and 3 compared to the controls. In mice exposed to BPA postnatally (group 2), the response to the OVA challenge was not affected (17), suggesting a critical window of exposure during gestation.

In the Midoro-Horiuti et al., 2010 (18) study, BALB/c mice were exposed one week before mating until lactation to 10 µg/ml BPA in their drinking water (around 900 µg/kg BW/day, Table 5). Offspring were

sensitized (i.p.) to OVA with Alum, and challenged with aerosolized OVA. Neonates from BPA-exposed mothers responded to this sensitization with higher serum titers of OVA-specific IgE compared with sensitized and challenged offspring from unexposed mothers. Eosinophilic inflammation in the BAL fluid was enhanced as well. Airway responsiveness to methacholine was enhanced in the OVA-sensitized neonates from BPA-treated mothers compared with neonatal mice from unexposed mothers (18).

These studies show that developmental BPA exposure enhances several measures that mirror respiratory allergy to OVA, with the exception of mice that were only postnatally exposed to BPA. These findings are consistent with the results Bauer et al reported in the mucosal sensitization model (12).

Conclusions and recommendations

Several experimental studies demonstrate some type of immune effects of BPA after developmental exposure. However, in most studies the doses used and which caused immunological effects were above those used to derive the current t-TDI. Exceptions to this 'high' dose include the publications of Ménard (10, 11) and Bauer (12), indicating adverse effects on the immune system at lower doses, and these were discussed in detail during the workshop.

From the animal studies considered, the Ménard study (10) of pre- and postnatal developmental exposure to BPA in rats showed a plethora of immune related parameters affected in offspring of dams given 5 µg/kg BW/day (10). This combination of effects was concluded by the workshop to represent an adverse health effect. Two other studies provide additional and supporting evidence of adverse immune effects in the same dose ranges (11, 12). At the lower dose of 0.5 µg/kg BW/day, an increase of anti-OVA IgG titers was observed, indicative of an impairment of oral tolerance induction (10), it was concluded at the workshop that the loss of oral tolerance indicated the possibility of an adverse effect at this lower dose as well. However, since no other immune parameters were assessed at the 0.5 µg/kg/d dose, there was no consensus on a more definitive statement on adversity at this dose level. Taken together, the developmental immune toxicity studies reviewed at the workshop provide novel information that warrants serious consideration in view of human safety.

The 5 µg/kg BW/day adverse immune effect dose in the Ménard (10) study was compared with the overall lowest BMDL10 in animal studies as determined by EFSA (2015) (1), which was 8960 µg/kg BW/day in the Tyl et al. (2008) study (2). From this BMDL10, EFSA (2015) derived an HED of 609 µg/kg BW/day, leading to a t-TDI of 4 µg/kg BW/day using an uncertainty factor of 150. A direct comparison between a given observed adverse effect level with a calculated BMDL10 (2) is not possible. However, given the fact that the adverse effect level of 5 µg/kg BW/day is much lower than the Tyl et al (2008) BMDL10 of 8960 µg/kg bw (2), the workshop agreed by consensus that this new publication, in the context of related animal studies, clearly warrants reconsideration of the EFSA t-TDI.

The effects of BPA on the developmental immune system are consistent with the general concept and weight of evidence demonstrating the relatively high sensitivity of the developing immune system to perturbation by various toxicants, the consequences of which are often not discerned until later in life (21). When considered in their totality, these studies suggest that current regulatory hazard and risk assessment strategies may not give sufficient attention to possible developmental immunotoxicity of chemicals (21). The Extended One Generation Toxicity Study (OECD-TG-443) is the only OECD guideline based study that provides an opportunity for developmental immunotoxicity testing. However this study, and especially its immunotoxicity and neurotoxicity cohorts, is currently only

carried out in exceptional cases at higher tonnage production levels under the REACH legislation. Evidence is accumulating that points to reconsideration of regulatory requirements for developmental immunotoxicity testing. With regard to BPA specifically, the workshop agreed by consensus that the information from these new publications, in the context of related animal studies, clearly warrants reconsideration of the 2015 EFSA t-TDI.

Table 1: Study 1 - protocol of oral tolerance and systemic immunization – dose response BPA (10)

Age of Wistar rats							
	G15 to 21 d	45 d	52 d	66 d	73 d		
Day of oral tolerance induction		P0	P7	P21	P28		
OVA-tolerized	0, 0.5, 5 or 50 µg BPA/kg/day	20 mg gavage	OVA	OVA in CFA 100 µg s.c.	OVA boost 100 µg s.c.	Euthanasia	
OVA-immunized	0, 0.5, 5 or 50 µg BPA/kg/day	Gavage, vehicle		OVA in CFA 100 µg s.c.	OVA boost 100 µg s.c.	Euthanasia	

Ménard et al., FASEB 2014 (10). G15: gestational day 15; P: protocol day; CFA: complete Freund Adjuvant; s.c.: subcutaneous

Table 2: Study 2 - protocol of oral tolerance and oral immunization – single dose BPA (10)

Age of Wistar rats							
	G15 to 21 d	45 d	52 d	59, 61, 63, 65 d	67 d		
Day of oral tolerance induction		P0	P7	P14, 16, 18, 20, 22	P22 + 1h		
OVA-tolerized	0 or 5 µg BPA/kg/day	20 mg gavage	OVA	OVA in CFA 100 µg s.c.	Gavage 50 mg OVA	Euthanasia	
OVA-immunized	0 or 5 µg BPA/kg/day	Gavage, vehicle		OVA in CFA 100 µg s.c.	Gavage 50 mg OVA	Euthanasia	

Ménard et al., FASEB 2014 (10). G15: gestational day 15; P: protocol day; CFA: complete Freund Adjuvant; s.c.: subcutaneous

Table 3: Study 3 - protocol of oral tolerance and systemic immunization –single dose BPA, juvenile challenge (11)

Age of Wistar rats							
	G15 to 21 d	25 d	32 d	46 d	53 d		
Day of oral tolerance induction		P0	P7	P21	P28		
OVA-tolerized	0 or 5 µg BPA/kg/day	20 mg gavage	OVA	OVA in CFA 100 µg s.c.	OVA boost 100 µg s.c.	Euthanasia	
OVA-immunized	0 or 5 µg BPA/kg/day	Gavage, vehicle		OVA in CFA 100 µg s.c.	OVA boost 100 µg s.c.	Euthanasia	

Ménard et al., PloS one 2014 (11). G15: gestational day 15; P: protocol day; CFA: complete Freund Adjuvant; s.c.: subcutaneous

Table 4: Results of the animal studies Ménard studies and Bauer

Model	Immune parameters affected at LOEL	LOEL	Considered by EFSA (1)
Oral tolerance model Ménard: study 1 (10)	<ul style="list-style-type: none"> • Increase of anti-OVA IgG in OVA-tolerized rats 	0.5 µg/kg BW/day	(-)
Oral immunization model Ménard: study 1 (10)	<ul style="list-style-type: none"> • IgG titers were significantly increased 	5 µg/kg BW/day	(-)
Oral challenge protocol Ménard: study 2: (OVA-tolerized) (10)	<p>Spleen</p> <ul style="list-style-type: none"> • increased cell proliferation • increased IFNγ • increased activated T lymphocytes (CD4+CD44highCD62Llow) <p>Colon</p> <ul style="list-style-type: none"> • increased inflammation • increased neutrophil infiltration • increased IFNγ and IL10 • decreased TGFβ 	5 µg/kg BW/day	(-)
Oral challenge protocol Ménard: study 2: (OVA-immunized) (10)	<ul style="list-style-type: none"> • increased IFNγ secretion (spleen) 	5 µg/kg BW/day	(-)
Oral tolerance model Ménard: study 3 (11)	<ul style="list-style-type: none"> • decrease IFNγ secretion (spleen) 	5 µg/kg BW/day	(-)
Oral immunization model Ménard: study 3 (11)	<ul style="list-style-type: none"> • decrease in IFNγ secretion (spleen and MLN) • decrease in helper T cells (spleen and MLN) • decrease in regulatory T cells (spleen and MLN) • decrease in dendritic cells (spleen and MLN) 	5 µg/kg BW/day	(-)
Host resistance model Ménard: study 4 (11)	<ul style="list-style-type: none"> • 1.5-fold increase in <i>N. brasiliensis</i> living larvae • decreased Neutrophil Infiltration • increases susceptibility to <i>N. brasiliensis</i> parasitic infection by deregulating Th1/Th2 cytokines profile in infected intestinal mucosa. (IL13, 4, 10 GRO/KC, IFNγ) 	5 µg/kg BW/day	(-)
Respiratory allergy model Bauer: mucosal sensitization model (12)	<ul style="list-style-type: none"> • enhanced lymphocytic infiltration and lung inflammation (in females only). 	>50 µg BPA/kg BW/day	(+)
Respiratory allergy model Bauer: systemic sensitization model (12)	<ul style="list-style-type: none"> • dampened lung eosinophilia • reduced OVA-specific IgE 	0.5 µg BPA/kg BW/day	(+)

Table 5. Overview of protocols from developmental immunotoxicity animal studies of BPA

Model	Protocol experimental immune model	Species	BPA: exposure period	BPA: exposure route and dose
Host resistance model Ménard: study 4 (11)	Parasite Infection: age day 25, day 0 s.c. injection <i>N. brasiliensis</i> . Day 7 Euthanasia, day 14 living larvae counting	Wistar female rats	GD15 - PND21	Oral Gavage, 0 or 5 µg/kg BW/day
Respiratory allergy model Bauer: mucosal sensitization model (12)	sensitization: age 6-10 weeks i.t. on days 0, 1, and 2 with 100 µg OVA, with, or without, 100 ng LPS. challenge: twice per day on days 14, 15, and 16 with aerosolized 1% OVA, 48 h later AHR assessment and euthanasia	C57Bl/6 mice	GD6 - PND21	Gavage, 0; 0.5; 5; 50 or 500 µg BPA/kg/day.
Respiratory allergy model Bauer: systemic sensitization model (12)	sensitization: age 6-10 weeks ip sensitized days 0 and 4 with PBS or 100 µg OVA in PBS + Alum challenged: once (1 h), on day 11, with aerosolized 1% OVA, 48h later AHR assessment and euthanasia	C57Bl/6 mice	GD6 - PND21	Gavage, 0; 0.5; 5; 50 or 500 µg BPA/kg/day.
Food allergy model Nygaard et al., 2015 (13)	immunization: age 4 weeks: intergastric gavage of 5.7 mg lupin extract (LE) + 10 µg cholera toxin (CT) as adjuvant on days 0, 1, 2, 7, 21 and 28. challenge: day 35 the mice were challenged with either a single dose of 5 mg LE i.p. or 25 mg LE divided on two i.g. administrations given 30 min apart. Anaphylaxis assessment, day 35 euthanasia	C3H/HeJ	Mating - end experiment	Drinking water: 0; 1; 10 or 100 µg/ml Calculated BPA: GD8: 180 µg/kg BW/day; 1.81 or 18.8 mg/kg BW/day; PGW2: 340 µg/kg BW/day; 3.46; 35 mg/kg BW/day; PGW 4: 230 µg/kg BW/day, 2.27, 22.17 mg/kg BW/day)**
Oral tolerance model Nygaard et al., 2015 (13)	tolerance: age 3 weeks lupin i.g. gavage (5 mg) age 4 weeks: i.g. of 5.7 mg lupin extract (LE) + 10 µg cholera toxin (CT) as adjuvant on days 0, 1, 2, 7, 21 and 28. challenge: day 35 the mice were challenged with either a single dose of 5 mg LE i.p. or 25 mg LE divided on two i.g.	C3H/HeJ	Mating - end experiment	Drinking water: 0; 1; 10 or 100 µg/ml Calculated BPA: GD8: 180 µg/kg BW/day; 1.81 or 18.8 mg/kg BW/day; PGW2: 340 µg/kg BW/day; 3.46; 35 mg/kg BW/day;

	administrations given 30 min apart. Anaphylaxis assessment, day 35 euthanasia			PGW 4: 230 µg/kg BW/day, 2.270, 22.17 mg/kg BW/day)**
Host resistance model Roy et al., 2012 (14)	Infection: age 6-8 weeks i.n. infected with Influenza A virus. Lung tissue harvested: day 3,7 and 10 after infection	C57BL/6 mice	GD6 - PND 21	Oral gavage, 0 or 50 µg/kg BW/day
Respiratory Allergy model Petzold et al., 2014 (15)	sensitization: age: 6-week old offspring were immunized i.p. with OVA, 20 mg + ALUM day 1 and 14 challenge: 20 mg OVA intranasally (i.n.) on days 14-16 and 21-23, AHR assessment and euthanasia.	BALB/c mice	Exposure all groups one week before mating, (discontinued during the mating period of 1 week) then: 1. GD0 - PND0 2. GD0 - PND21 3. GD0 - lifelong exposure	Drinking water, 0 or 5 µg/ml (+/- 450 µg/kg BW/day)*
Respiratory Allergy model O'Brien et al., 2014 (16)	sensitization: twelve-week-old mice OVA + Alum i.p. injection. challenge: One week after sensitization, challenged twice, with 24 h in between, by exposure to an aerosol of 3% OVA in PBS for 20 min, 24 h after the second sensitization euthanasia.	BALB/c mice	two weeks before mating - PND21	Diet: 0; 50 ng; 50 µg or 50 mg BPA/kg of rodent chow (+/- 7.5 ng, 7.5 µg, or 7.5 mg BPA/kg BW/day).
Respiratory allergy model Nygaard et al., 2015 (13)	sensitisation: 10 mg OVA i.p. without adjuvant at postnatal day (PND) 4 and 18. challenge: PND 25 with 10 mg OVA intranasally (i.n.). PND 30 euthanasia.	BALB/c mice	Mating - end lactation period	Drinking water: 0; 10 or 100 µg/ml Calculated BPA: GD8: 1.41 or 13.7 mg/kg BW/day and PGW2: 4.50 or 43.7 mg/kg BW/day)**
Respiratory Allergy model Nakajima et al. (2012) (17)	sensitization: age PND 4 ip injection 5 µg OVA + alum on PND 4. challenge: aerosolized 3% OVA for 10 min on PNDs 18, 19, 20 and AHR assessment day 22 and euthanasia	BALB/c mice	1: one week prior to gestation - PND25; 2: PND2 - PND25; 3: one week prior to gestation - PND2	Drinking water, 0 or 10 µg/ml (+/- 900 µg/kg bw/day)*

Respiratory Allergy model Midoro-Horiuti et al., 2010 (18)	sensitization: age PND4: ip injection 5 µg OVA + alum on PND 4. challenge: aerosolized 3% OVA for 10 min on PNDs 13, 14, and 15. AHR assessment PND 17 and euthanasia.	BALB/c mice	one week before mating - lactation	Drinking water, 0 or 10 µg/ml (+/- 900 µg/kg bw/day)*
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*For chronic studies, a default factor of 0.05 for rats and 0.09 for mice should be used, e.g. 1 mg/L in water is equivalent to a dose of 0.09 mg/kg bw per day in mice (EFSA, Administration of test substances in drinking water). ** calculated BPA reported in the study at GD8 (Gestational Day); PGW2 or PGW 4 (Post Gestational week 2 or 4, respectively).

Table 6. Overview of results from the animal studies on immunotoxicity of BPA considered by the workshop participants OR at the workshop

Model	Immuunparameters affected (effected dose)	LOEL	Considered by EFSA (1)
Food allergy model Nygaard et al., 2015 (13)	<ul style="list-style-type: none"> • increase in splenocyte cytokines ((IL-13, IFNγ and IL2) • decrease in mast cell protease (MMCP)-1 	18.8-35 mg/kg BW/day	(no)
Oral tolerance model Nygaard et al., 2015 (13)	<ul style="list-style-type: none"> • decrease in mast cell protease (MMCP)-1 	18.8-35 mg/kg BW/day *	(yes)
Host resistance model Roy et al., 2012 (14)	<ul style="list-style-type: none"> • decreased the extent of infection-associated pulmonary inflammation • decreased TNF-α • decreased IFN-γ • decreased chemokines RANTES (CCL5), • decreased IP-10 (CXCL10)) • decreased iNOS in lung tissue 	50 μ g/kg BW/day	(yes)
Respiratory Allergy model Petzold et al., 2014 (15)	Group 3 (lifelong exposure): <ul style="list-style-type: none"> • increased eosinophilic inflammation in the lung • increased airway hyperreactivity in OVA-sensitized adult mice. • increased antigen-specific serum IgE levels Group 1 and 2 no effects.	450 μ g/kg BW/day	(no)
Respiratory Allergy model O'Brien et al., 2014 (16)	<ul style="list-style-type: none"> • increase in anti-OVA IgE • increase IL13 (spleen) • increase IFNγ increased 	50 μ g BPA/kg of rodent chow (=7.5 μ g/kg BW/day)	(no)
Respiratory allergy model Nygaard et al., 2015 (13)	<ul style="list-style-type: none"> • increase in eosinophil numbers in BAL fluid 	13.7-43.7 mg/kg BW/day	(no)
Respiratory Allergy model Nakajima et al. (2012) (17)	Group 1 (pre and postnatal exposure) and 3 (prenatal exposure): <ul style="list-style-type: none"> • increased airway hyperreactivity • increased eosinophil numbers in BAL fluid Group 2 no effect (postnatal exposure).	900 μ g/kg BW/day	(yes)
Respiratory Allergy model Midoro-Horiuti et al., 2010 (18)	<ul style="list-style-type: none"> • increase in IgE anti-OVA • increased Eosinophilic inflammation in BAL fluid • Airway responsiveness (AHR with Methacholine) 	900 μ g/kg BW/day	(yes)

BAL broncho-alveolar lavage fluid, i.p. intraperitoneal, MLN mesenteric lymph nodes, *calculated BPA reported in the study at GD8; PGW2 and PGW 4 (13).

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