



## Developmental immunotoxicity testing of 4-methyl anisole



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### ABSTRACT

The developmental immunotoxicity of 4-methyl anisole (4MA) was investigated in the rat. Four study designs were used, with either pre-mating or post-weaning onset of exposure, continued to postnatal day 50, and with or without additional oral gavage of pups from postnatal day 10 onward. Reduced litter size (benchmark dose lower confidence limit (BMDL) 80 mg/kg bw/day) was the most sensitive developmental parameter, with pup relative organ weight effects observed at similar BMDLs, in the absence of maternal toxicity. Eosinophil numbers were reduced at lower doses (BMDL 16 mg/kg bw/day). KLH challenge resulted in increased IL-13 and TNF- $\alpha$  responses, and variably reduced IgG production (BMDL 27 mg/kg bw/day). T<sub>4</sub> levels were reduced by 11% at maximum with a BMDL of 73 mg/kg bw/day. Differences between exposure cohorts were limited and were considered to be without biological significance. This study shows that 4MA induces developmental immunotoxicity at doses below those inducing developmental and general toxicity. These observations being independent of the study designs applied suggest that the post-weaning period, included in all designs, is the most relevant sensitive period for inducing 4MA mediated developmental immunotoxicity. Moreover, this study stresses the importance of including developmental immunotoxicity testing by default in regulatory toxicology.

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### 1. Introduction

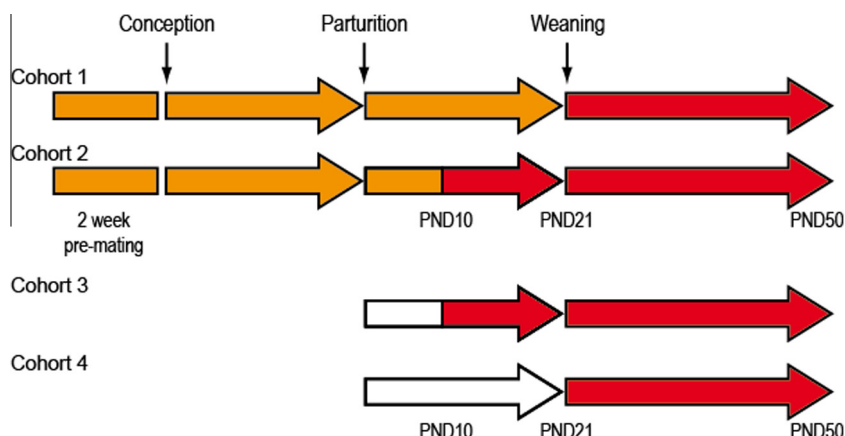
Developmental immunotoxicology is a relatively neglected area in regulatory toxicity testing. The recent acceptance of the OECD TG 443 Extended One generation Reproductive Toxicity Study (EOGRTS), including a specific cohort for immune testing provides a significant step forward (OECD, 2011, TG 443), but this test may only be required at high tonnage levels under the European REACH regulation for chemical safety. Some structural immune parameters such as lymphoid organ weights and subset organ cellularities are also monitored in regular acute and subchronic toxicity testing. However, the developing immune system may be more vulnerable to toxic insults, which warrants specific testing in developmental phases. In addition, structural immune system parameters, as opposed to functional immune parameters, may appear normal in the presence of affected immune responsiveness to an immunological challenge. The realization that developmental immune toxicity testing may be important in chemical hazard identification and risk assessment is further supported by increasing trends of immune-related diseases in man that have an early onset, such

as allergies, asthma and a variety of autoimmune diseases. Earlier studies have indicated that the developing immune system may have a specific sensitivity to compound exposure (Miller et al., 1998; Chapin et al., 1997; Gehrs et al., 1997; Smialowicz et al., 1988). We have previously tested several compounds in several developmental exposure designs, studying general, developmental and immunotoxic effects (Tonk et al., 2011a,b, 2010, 2013a,b, 2012). Overall, it appeared that developmental immune parameters were or were among the most sensitive parameters studied. As a consequence, developmental immune parameters often (co-)determined the overall NOAEL, and, if tested in a regulatory setting, would have had an impact on the derivation of threshold levels for human exposure such as TDI or ADI.

A variety of relevant study designs can be envisaged for developmental immunotoxicity testing. Provisional comparisons of generational exposure from the pre-mating phase to offspring adulthood versus juvenile exposure studies indicate that both exposure scenarios have their advantages in detecting developmental immunotoxicity (Tonk et al., 2011a,b, 2013a,b). As generational exposure such as in the EOGRTS includes long term continuous exposure, effects caused during any phase of the entire developmental window can be detected. However, adaptation to exposure and prenatal programming can potentially influence postnatal sensitivity. A juvenile exposure design may show a higher sensitivity during early postnatal development but is also

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**Fig. 1.** Study design. Orange: maternal exposure via gavage; red: direct F<sub>1</sub> exposure via gavage. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

limited to this particular developmental window. The relative sensitivity of the juvenile period needs further study in order to clarify whether a specific testing protocol is warranted. Such a specific protocol may or may not need to include direct dosing of pups during lactation to ensure pup exposure, with or without direct exposure of the pregnant or lactating dams.

In the present study we assessed a series of developmental immune parameters in generational and juvenile exposure protocols with and without direct pup exposure during lactation using 4-methylanisol (4MA) as a test compound. 4MA is a food flavoring agent which naturally can be found in oil of ylang ylang used in fragrances. Currently 4MA is used a.o. in cleansing agents, air care products, biocidal products and scented toys. 4MA is soluble in apolar solvents with a Pow around 2.7, and after topical exposure 12% of the applied dose is excreted in urine, suggesting that systemic exposure does occur, possibly also via the oral route (ECHA). Therefore, lactational exposure may be a relevant route of weanling exposure. This compound has been shown to reduce spleen and thymus weight in a repeat dose toxicity study (OECD, 2008, TG 407), and caused pup mortality and reduced pup weight in a reproductive and developmental toxicity screening study (OECD, 1995, TG 421). Based upon the latter study, in Europe 4MA received an H361 classification for suspicion of damaging fertility or the unborn child (ECHA). The overall NOAEL was 100 mg/kg bw/day in both these studies. Thus, given that 4MA affected developmental as well as immune parameters in earlier studies, this compound was considered a relevant candidate for dedicated developmental immunotoxicity testing. Moreover, direct pup exposure via the oral route was included in this study in order to establish whether this developmental window might be particularly sensitive for 4MA induced effects.

## 2. Materials and methods

### 2.1. Animals

The animal experiment was carried out at Intravacc, Bilthoven, The Netherlands. Animal care and use were in accordance with the general principles of governing the use of animals in experiments of the European communities (Directive 86/609/EEC) and with Dutch-specific legislation (The Experiments on Animals Act). Parental (F<sub>0</sub>) Wistar outbred rats were obtained from Harlan, The Netherlands. Animals were given a two week acclimatization period before the start of the experiment and housed in groups in macrolon cages with 12:12 h light:dark cycle, maintained at 22 ± 2 °C, 45–90% humidity on a commercial rodent diet (Rat & Mouse No. 3 breeding diet, RM3, SDS Special Diets Services,

Witham, England). F<sub>0</sub> animals were mated at a ratio of 2 females:1 male. The day of sperm detection in the vaginal smear was considered day 0 of gestation and mated F<sub>0</sub> females were housed individually for the birth and rearing of their young. The morning after birth was considered postnatal day (PND) 1, litters were not standardized and pups were weaned on PND 21.

### 2.2. Test compound and exposure

4-Methylanisol (4MA), CAS 104-93-8, with a labeled purity of 99% was purchased from Aldrich and dissolved in laboratory-grade corn oil (CAS 8001-30-7, MP Biomedicals). The maximum period for which each preparation was used was 7 days. During exposure, animals were administered a daily oral dose of 0 (vehicle control), 8, 16, 32, 64, 125, or 250 mg/kg bw/day 4MA in 5 ml vehicle per kg bw except for juvenile animals (PND 10–21) for which a dosing volume of 10 ml per kg bw was used. Bodyweight were monitored twice weekly in F<sub>0</sub> animals and in F<sub>1</sub> animals after weaning, in pregnant dams on gestation day (GD) 0, 4, 7, 10, 13, 17, 21, lactation day (LD) 1, 4, 7, 10, 13, 17, 21 and pups on PND 1, 4, 7, 10, 13, 17, and 21.

A schematic diagram of the study design is shown in Fig. 1. In cohort 1 F<sub>0</sub> females are exposed from 2 weeks pre-mating, during mating, gestation and lactation and pups receive a vehicle from PND 10 to 21 and are individually exposed from PND 21 (weaning). Cohort 2 are exposed similarly as cohort 1 except maternal exposure stops at LD10 and F<sub>1</sub> pups are directly exposed from PND 10. In cohort 3 and 4 animals there is no maternal exposure, cohort 3 F<sub>1</sub> is exposed directly from PND 10 while cohort F<sub>1</sub> animals are directly exposed from PND 21 (receiving a vehicle from PND 10 to 21).

### 2.3. Effects assessment

F<sub>1</sub> males were examined daily for the onset of preputial separation (PPS) as an indicator for the onset of puberty from PND 31 until complete separation or necropsy (PND 50).

Subsets of F<sub>1</sub> males ( $n = 6$ /dose group) originating from different litters, were evaluated for the effects of 4MA exposure on PND 50. Terminal body weight were recorded, EDTA blood was collected and one femoral shaft was flushed with 4 ml Impulse Cytophotometer (ICP) solution (Tonk et al., 2010). The bone marrow cell suspension and EDTA blood were kept at 4 °C until automated analysis using an ADVIA 120 Hematology System (Siemens) within 4 h. Liver, kidneys, spleen, thymus, adrenals, testes, heart and brain were removed and weighed. The right testis was frozen on dry ice and used for testis spermatid head count. The tunica alba was

removed, the testis homogenized in 20 ml phosphate buffered saline (PBS) with 0.1% triton X-100 using a Pro200 homogenizer and counted on a hemocytometer. T<sub>3</sub>, T<sub>4</sub>, and TSH levels were measured in blood using a MILLIPLEX Map Kit (Millipore) according to the vendor's protocol.

Immune assessments were performed as described previously. Single-cell splenocyte suspensions were examined for lymphocyte subpopulation distributions using a FACSCalibur flow cytometer (BD Biosciences). Adherent splenocytes were exposed to lipopolysaccharide (LPS) (SIGMA) for 24 h after which supernatants were used to measure nitric oxide (NO) using the Griess reaction and tumor necrosis factor (TNF) $\alpha$ .

A separate group of F<sub>1</sub> males ( $n = 6/\text{dose group}$ ) were immunized with two subcutaneous injections of 0.2 ml of 5 mg/ml keyhole limpet hemocyanin (KLH) (Pierce) at PND 21 and 35. Primary IgM (PND 26), primary IgG (PND 35) and secondary IgM and IgG (PND 40) responses to KLH were determined using an anti-KLH IgM- or IgG-specific ELISA. A delayed-type hypersensitivity response was assessed using a challenge with 10 mg/ml KLH in 0.01 ml of saline injected into one ear and an equivalent volume of saline injected in the other ear on PND 45. The DTH response was measured 24 h later using a digital caliper. The KLH-immunized F<sub>1</sub> males were euthanized on PND 50, spleens were removed and splenocyte suspension re-stimulated with 100  $\mu\text{g}/\text{ml}$  KLH for 96 h. Collected supernatant were assessed for IL-4, IL-6, IL-13, IL-10, TNF $\alpha$ , and IFN $\gamma$  using a MILLIPLEX Map Kit (Millipore) according to the vendor's protocol.

## 2.4. Data analysis

The dose–response data were analyzed using the benchmark dose (BMD) approach with the PROAST software (Slob, 2002) ([www.rivm.nl/proast](http://www.rivm.nl/proast)) as described previously (Tonk et al., 2012). Dose–responses models were fitted to the data, a benchmark response (BMR) was defined and the associated BMD was derived from the fitted model. BMRs used in this study were 5% for continuous data and 10% (extra risk) for quantal data as proposed by the European Food Safety Authority (EFSA) (EFSA, 2009). For continuous data, optimal models were selected from the exponential and Hill model families based on a goodness-of-fit test. The BMD used in the analysis was the geometric average of the BMDs derived for the different selected models. The 90%-confidence interval surrounding this BMD comprised of the lowest 5% lower confidence bound (BMDL) and the highest 95% upper confidence bound (BMDU) for the BMD estimated derived from the different models. The models are suitable for describing multiple subpopulations by the same model. When animals from the 4 cohorts are equally sensitive to 4MA the dose–response data can be described with a single model, potentially with parameter 'a' differing between models to account for diverse backgrounds. When cohorts are not equally sensitive, parameter 'b' differs between cohorts. The hypothesis that parameter 'a' and/or 'b' differ between cohorts was statistically tested using a likelihood ratio test. For the quantal data logistic, probit, log–logistic, log–probit, Weibull, gamma models and the linearized multistage model family were used. Intra-litter variations were taken into account using a beta-binomial distribution (quantal data) or using the bootstrap method to calculate the 90%-confidence interval surrounding the BMD (continuous data).

## 3. Results

### 3.1. Parental assessment

F<sub>0</sub> animals showed no adverse behavior or clinical signs except for F<sub>0</sub> females dosed with 125 or 250 mg/kg bw/day 4MA which

showed excess salivation after dosing. F<sub>0</sub> females showed dose-dependent increases in body weight throughout gestation and lactation, however the maximum response did not exceed 5% (Table 1).

### 3.2. F<sub>1</sub> assessment

No effects of 4MA were observed on fertility and reproductive performance (mating index, fertility/fecundity index, gestation index, precoital time, gestation time). The litter size was decreased at the highest dose level only (BMD 210 mg/kg bw/day 4MA, CI 80–224, max. response –24% at 250 mg/kg bw 4MA). A minimal decrease in body weight was observed in F<sub>1</sub> animals cohort 1 + 2 on PND 7 and F<sub>1</sub> animals cohort 1, 2 and 3 on PND 13, 17, and 21; however, the maximum response did not exceed 5% with no observed differences between cohorts (Table 1).

### 3.3. Organ weights

Dose-dependent increases were observed for absolute liver and kidney weights while thymus, heart and brain weights showed dose-dependent decreases. Relative liver and kidney weights were dose-dependently increased with maximum responses of +13% and +7%, respectively at the highest dose level. Relative thymus weight was dose-dependently decreased with a maximum response of –7%. No differences between cohorts were observed for any of the effects on organ weights (Tables 1 and 2).

### 3.4. Hormone analysis

T<sub>4</sub> levels were dose-dependently decreased in cohort 1, 2 and 4 animals only (Table 3) (cohort 1: BMD 110 mg/kg bw/day 4MA, CI 73–212, max. response –11%; cohort 2: BMD 454 mg/kg bw/day 4MA, CI 19–Inf, max. response –3%; cohort 4: BMD 164 mg/kg bw/day 4MA, CI 91–624, max. response –7%) (Fig. 2). No effects were observed on T<sub>3</sub> and TSH levels.

### 3.5. Immune assessment

#### 3.5.1. Hematology and bone marrow

Mean corpuscular volume (MCV) showed an increasing trend with a maximum response <5% (Table 4). Platelet counts and the number of eosinophils were dose-dependently decreased with maximum responses of –6% and –34%, respectively at the highest

**Table 1**  
Body weights, litter sizes, organ weights.

		BMD (%)		Max. response
		Point estimate	BMDL–BMDH	
Dam body weight	PND21	347.9	190.1–2067.0	4%
Litter size		210.7	80.2–224.7	–24%
Pup body weight	PND21	263.7	57.6–1366	–4.5%
<i>Organ weights</i>				
Liver (g)		119.2	85.15–191.9	11%
Liver (g/100 g BW)		104.5	85.1–132.3	13%
Kidney (g)		229.7	134.1–810.0	5%
Kidney (g/100 g BW)		193.8	141.2–306	7%
Adrenal (g)		–		
Adrenal (g/100 g BW)		–		
Testis (g)		–		
Testis (g/100 g BW)		–		
Heart (g)		292.7	158.21–1694.1	–4%
Heart (g/100 g BW)		–		
Brain (g)		273.6	253.72–466.7	–3%
Brain (g/100 g BW)		–		

**Table 2**  
Lymphoid organ weights and cellularities.

	BMD (%)		Max. response
	Point estimate	BMDL–BMDH	
Thymus (g)	154.16	91.90–410.2	–8%
Thymus (g/100 g BW)	189.7	105.4–769.8	–7%
Spleen (g)	113.5	69.41–262.4	–11%
Spleen (g/100 g BW)	130.2	78.2–328.7	–9%
Cellularity ( $10^7$ )	115.2	65.3–355.1	–10%
Cellularity ( $10^7$ /g)	–	–	–
<i>No. of cells per spleen (<math>10^7</math>)</i>			
T cell (CD3 <sup>+</sup> )	–	–	–
CD4 <sup>+</sup> CD8 <sup>–</sup>	–	–	–
CD4 <sup>–</sup> CD8 <sup>+</sup>	–	–	–
NK cell	–	–	–
B cell	–	–	–
<i>Relative no. of cells per spleen (<math>10^7</math>/g)</i>			
T cell (CD3 <sup>+</sup> )	–	–	–
CD4 <sup>+</sup> CD8 <sup>–</sup>	–	–	–
CD4 <sup>–</sup> CD8 <sup>+</sup>	–	–	–
NK cell	–	–	–
B cell	–	–	–
CD4 <sup>+</sup> /CD8 <sup>+</sup> ratio	–	–	–
T/B cell ratio	Cohort 1	–	–
	Cohort 2	–	–
	Cohort 3	123.3	0.0–Inf 11%
	Cohort 4	53.6	35.5–97.1 27%

**Table 3**  
Thyroid hormone levels.

<u>BMD (%)</u>			Max. response
	Point estimate	BMDL–BMDH	
<i>Thyroid hormones (pg/ml)</i>			
T <sub>3</sub>		–	
T <sub>4</sub>	Cohort 1	110.5	72.55–212
	Cohort 2	454.1	158.8–Inf
	Cohort 3	–	
	Cohort 4	164.1	91.3–624
TSH		–	–11%
			–3%
			–7%

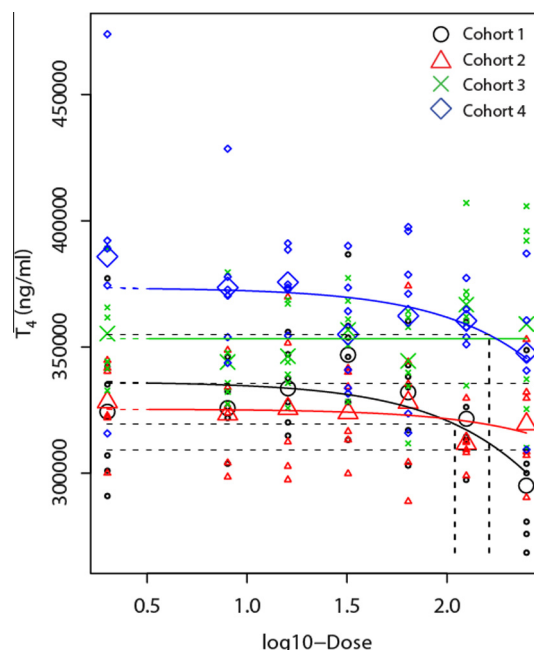
dose level (platelet count: BMD 215 mg/kg bw/day 4MA, CI 119–945, max. response –6%; eosinophils: BMD 25.5 mg/kg bw/day 4MA, CI 15.9–42.5, max. response –36%) (Fig. 3). No differences between cohorts were observed for any of the effects on hematological end points. No effects were observed on bone marrow cellularity.

### 3.5.2. Spleen

Both absolute and relative spleen weight showed a dose-dependent decrease with maximum responses of –11% and –9%, respectively at the highest dose level (Table 2). The number of cells per spleen showed a similar dose-dependent decrease. However, no effect was observed for the relative splenic cellularity (cells/gram spleen). No effects were observed on splenic lymphocyte subpopulations except for the T/B cell ratio which was dose-dependently increased in cohort 3 and 4 animals only (cohort 3: BMD 123 mg/kg bw/day 4MA, CI 0–Inf, max. response +11%; cohort 4: BMD 54 mg/kg bw/day 4MA, CI 35–97, max. response +27%) (Fig. 4).

### 3.5.3. Functional immune parameters

TNF- $\alpha$  production by LPS-stimulated adherent splenocytes was dose-dependently increased with a maximum response of +27% at 250 mg/kg bw 4MA (Fig. 5), while NO production was not affected by 4MA exposure (Table 5). KLH-specific parameters such as anti-KLH antibody responses and DTH showed no dose-dependent



**Fig. 2.** T<sub>4</sub> dose–response curves in four exposure protocols. Small points: individual observations; large points: group (geometric) means; horizontal dashed lines: 5% BMR; vertical dashed lines: BMDs associated with BMR.

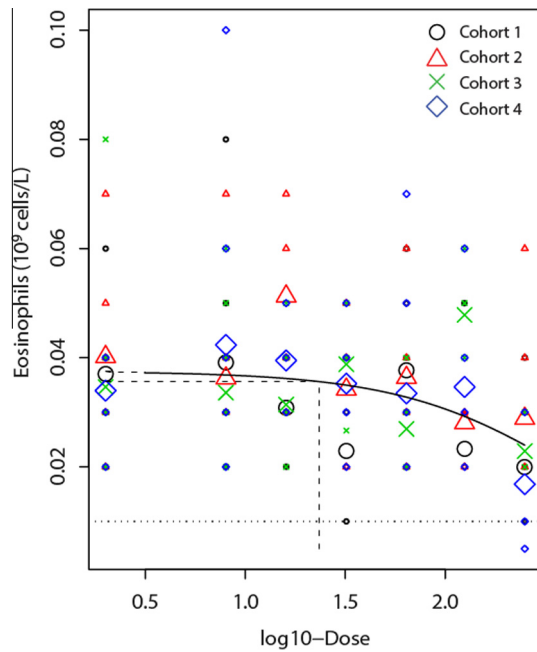
**Table 4**  
Hematology and thyroid hormones.

	BMD (%)		Max. response
	Point estimate	BMDL–BMDH	
<i>Red blood cell characteristics</i>			
RBC (10 <sup>12</sup> /l)	–		
HGB (mmol/l)	–		
HCT (%)	–		
MCV (fl)	743	439–2381	2%
MCH (f mol)	–		
MCHC (mmol/l)	–		
RDW (%)	–		
HDW (mmol/l)	–		
PLT (10 <sup>9</sup> /l)	215	119–945	–6%
MPV (fl)	–		
Reticulocytes (10 <sup>9</sup> /l)	–		
<i>White blood cell populations (10<sup>9</sup>/l)</i>			
WBC	–		
Neutrophils	–		
Lymphocytes	–		
Monocytes	–		
Eosinophils	25.5	15.9–42.5	–36%
Basophils	–		
LUC	–		
<i>Bone marrow</i>			
Cellularity (10 <sup>9</sup> /l)	–		

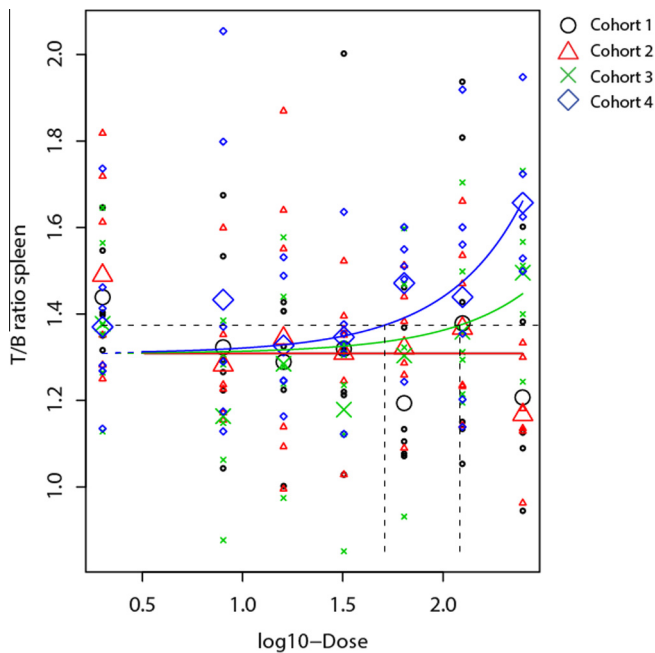
effects. For the anti-KLH IgG response on PND40 a decreasing dose–response model was selected by the fitting algorithm, however no convergence was reached for the exponential model fit probably because the noise within dose groups was large compared to the differences in response. Therefore, the data leave ambiguity about the dose–response relationship (Fig. 6).

For KLH-stimulated cytokine production, no effects were observed on IL-2, IL-4, IL-10 and IFN- $\gamma$ , while IL-6 levels were below the detection limit. KLH-stimulated IL-13 (Fig. 7) and TNF- $\alpha$  levels (Fig. 8) were dose-dependently increased with maximum responses of +46% and +25%, respectively at the highest dose level.





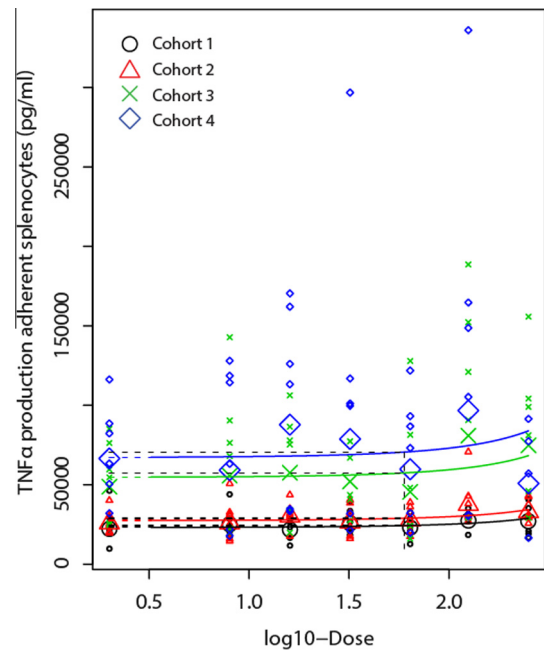
**Fig. 3.** Combined dose-response of eosinophil counts in all exposure protocols. Small points: individual observations; large points: group (geometric) means; horizontal dashed line: 5% BMR; vertical dashed line: BMD associated with BMR; dotted line: detection limit (0.01).



**Fig. 4.** T/B ratio in spleen, dose-response curves in four exposure protocols. Small points: individual observations; large points: group (geometric) means; horizontal dashed line: 5% BMR; vertical dashed lines: BMDs associated with BMR.

#### 4. Discussion

The prevalence in children of early onset diseases related to malfunctioning of the immune system, such as asthma, allergies and a host of autoimmune diseases, including e.g. inflammatory bowel disease and diabetes, are steadily increasing in western societies (Dietert, 2011). Among a wide variety of suggested causes are



**Fig. 5.** TNF $\alpha$  production in splenocytes, dose-response curves in four exposure protocols. Small points: individual observations; large points: group (geometric) means; horizontal dashed lines: 5% BMR; vertical dashed line: BMD associated with BMR.

**Table 5**

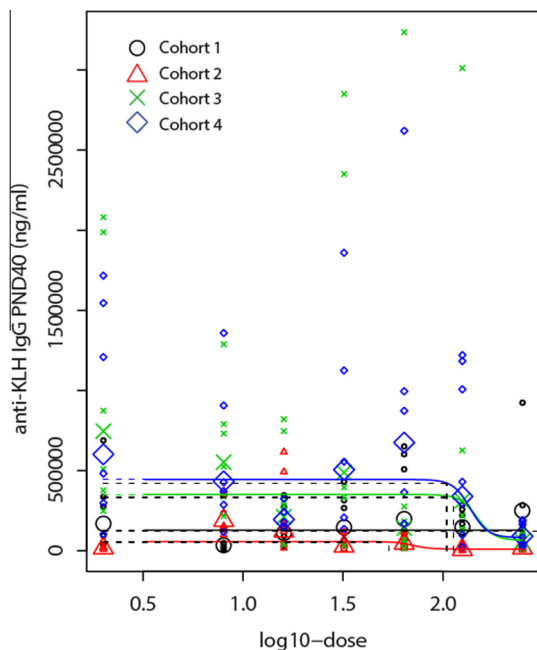
Functional immune parameters.

		BMD (%)		Max. response
		Point estimate	BMDL–BMDH	
<i>Anti-KLH antibody response (ng/ml)</i>				
IgM – PND 26		–		
IgM – PND 40		–		
IgG – PND 35		–		
IgG – PND 40 <sup>a</sup>	Cohort 1	–		
	Cohort 2	53.8	26.6–79.6	–82%
	Cohort 3	112.8	59.8–175.0	–81%
	Cohort 4	104.4	46.3–182.9	–81%
DTH ( $\Delta$ mm)		–		
<i>KLH-stimulated cytokine production</i>				
IL-2 (pg/ml)		–		
IL-4 (pg/ml)		–		
IL-6 (pg/ml)		ND		
IL-13 (pg/ml)		34.8	19.9–87.6	46%
IL-10 (pg/ml)		–		
TNF $\alpha$ (pg/ml)		57.0	31.1–217.9	25%
IFN- $\gamma$ (pg/ml)		–		
<i>Adherent cells</i>				
NO (nmol/mg protein)		–		
TNF- $\alpha$ (pg/mg protein)		53.8	30.0–175.2	27%

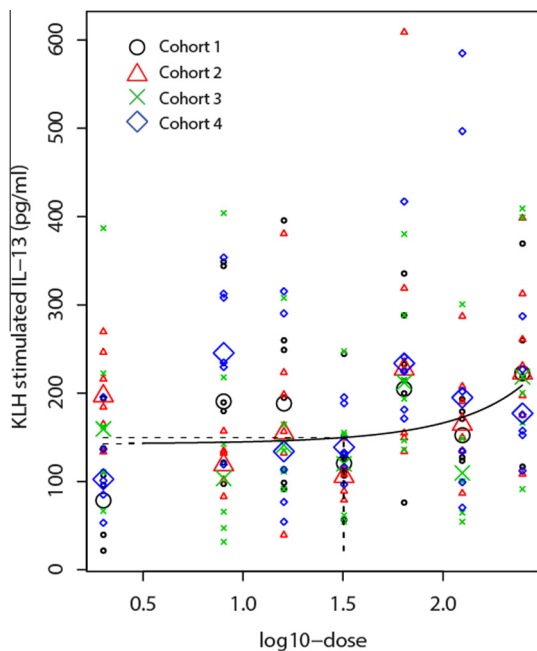
ND: values below detection limit.

<sup>a</sup> Data leave ambiguity about the dose-response relationship.

pre- and early postnatal developmental exposures to chemicals. Although chemical exposures have increased alongside increases in disease prevalences, there is much uncertainty about possible causative relationships between chemical exposures and immune related diseases, as current testing paradigms for chemicals and drugs do not regularly include testing for developmental immunotoxicity. Given the impact of the aforementioned diseases on public health, both in terms of wellbeing and cost, regular testing for developmental immunotoxicity may be considered (Piersma et al., 2012). This suggestion would be strengthened if

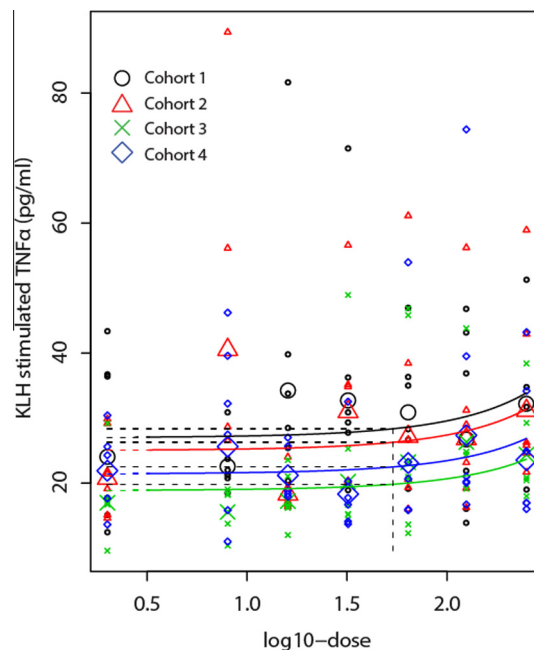


**Fig. 6.** Anti-KLH IgG at PND40, dose-response curves in four exposure protocols. Small points: individual observations; large points: group (geometric) means; horizontal dashed lines: 5% BMR; vertical dashed lines: BMDs associated with BMR.



**Fig. 7.** Combined dose-response KLH-stimulated IL13 production in all exposure protocols. Small points: individual observations; large points: group (geometric) means; horizontal dashed line: 5% BMR; vertical dashed line: BMD associated with BMR.

experimental studies would show a relatively high sensitivity of the developing immune system in reproductive and developmental toxicity studies. Several studies heretofore have indicated that the developing immune system is relatively vulnerable to chemical exposures. Examples include a variety of chemical structures such as methylmercury (Tonk et al., 2010), diethyl hexyl phthalate (Tonk et al., 2012), TCDD (Gehrs et al., 1997) and methoxychlor



**Fig. 8.** KLH stimulated TNF $\alpha$ , dose-response curves in four exposure protocols. Small points: individual observations; large points: group (geometric) means; horizontal dashed lines: 5% BMR; vertical dashed line: BMD associated with BMR.

(Chapin et al., 1997). The present study adds observations on developmental toxic effects of 4MA.

4MA caused some classical developmental effects, and at lower dose levels effects on the developing immune system were found, all at doses without overt toxicity. There were only few differences in immune parameter changes between the four study designs. No clinical signs were observed except for transient salivation, which may have been induced by the unpleasant taste of the test substance and/or by local irritation of the upper digestive tract. This finding was not considered as a sign of systemic toxicity. As to reproductive and developmental effects, the observed decrease in litter size at the highest dose level (BMDL 80 mg/kg bw/day) is in line with previous studies reporting a NOAEL of 100 mg/kg bw/day (ECHA). Effects on pup relative organ weight (liver, kidney, thymus, spleen) are similar to the effects observed in a repeated dose 28-day oral toxicity study and are found in the same order of magnitude (BMDLs 78–141 mg/kg bw/day) (ECHA). Immune parameters were affected at lower exposure levels with BMDLs calculated at 17 and 19 mg/kg bw/day for the number of eosinophils in blood and KLH-specific IL-13 production, respectively. Also, TNF- $\alpha$  production by adherent splenocytes and KLH-specific TNF- $\alpha$  production were affected with BMDLs around 30 mg/kg bw/day. Effects on eosinophil counts and TNF- $\alpha$  production by adherent splenocytes can be indicative of effects on the innate immune system. A decreased eosinophil count was previously observed after ethanol (EtOH) exposure in an EOGRS (Tonk et al., 2013) while TNF- $\alpha$  production by adherent splenocytes was affected after exposure to DEHP (adult exposure PND 50–90) (Tonk et al., 2012), EtOH (juvenile exposure protocol and EOGRS) (Tonk et al., 2013a,b), and DOTC (juvenile exposure only) (Tonk et al., 2011). In the present study, the 4MA induced increases in KLH-specific IL-13 and TNF- $\alpha$  production indicates an effect on the developing immune system. IL-13 is a Th<sub>2</sub>-type cytokine associated with physiological changes induced by allergic inflammation in many tissues. TNF- $\alpha$  promotes general inflammatory responses. This study adds to the evidence showing that developmental immune parameters can be among the most sensitive in

developmental exposure designs. Earlier studies from our and other laboratories have shown similar relative sensitivities. This includes studies with a variety of compounds, such as lead, tributyltin oxide, TCDD and methoxychlor (Miller et al., 1998; Chapin et al., 1997; Gehrs et al., 1997; Smialowicz et al., 1988). This growing body of evidence warrants serious consideration of regular developmental immune parameter testing in regulatory reproductive toxicity studies. The relatively novel OECD TG 443 EOGRS provides a first regulatory landmark for studying such parameters (OECD, 2011, TG 443).

There were only few differences in responses between the cohorts. The anti-KLH IgG dose-responses, although statistically significantly different from no effect, may not be biologically relevant given the wide variation within dose groups. The splenic T/B ratio was increased in cohort 4 only; however, this effect was not supported by 4MA induced effects on individual subpopulations in spleen. Assessment of the thyroid hormone levels in blood suggested a differential sensitivity for 4MA induced effects between the cohorts. The magnitudes of the responses (–11% at maximum) were low and the toxicological significance of these findings is therefore unclear. The lowest BMDLs calculated for the 4MA induced increases in T<sub>4</sub> levels was found in cohort 1 and was in the same order of magnitude as the general toxicity and developmental effects (73 mg/kg BW/day). These changes could be the result of induced hepatic glucuronyl transferases resulting in increased catabolism of the thyroid hormone. Overall, no toxicologically important differences were observed in sensitivity due to different exposure regimens in the four cohorts, with only modest effects found and no consistency in cohort sensitivity. Earlier studies have indicated that the exposure window, developmental versus juvenile, as well as added direct oral pup exposure versus lactational exposure only, might affect the outcome of the study. For instance, DOTC and ethanol, after generational exposure showed a lower incidence and magnitude of developmental immune effects as compared to juvenile exposure only (Tonk et al., 2011a,b, 2013a,b). This was suggested to be attributable to induction of tolerance by increased metabolism of the compound induced in the prenatal phase, reducing the susceptibility in the juvenile phase. Apparently, the major window of sensitivity to these compounds is in the postnatal juvenile phase. Moreover, as added direct lactational exposure (cohorts 2 and 3 in this study) did not alter the overall hazard assessment, this indicates that either the weaning period is not the most sensitive period for 4MA induced toxicity, or the exposure via lactation is sufficient to generate the effects observed. Ongoing work examining internal exposure during weaning in the various cohorts in this study will shed further light on this issue. For general screening of novel or existing compounds however, and in view of the fact that in all study designs several developmental immune parameters were responsive, it seems prudent to study generational exposure in order to cover all possibly relevant exposure windows within the

reproductive cycle, such as is described in the OECD TG 443 EOGRS.

### Conflict of interest

The authors report no conflict of interest.

### Transparency Document

The [Transparency document](#) associated with this article can be found in the online version.

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