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# Immunomodulation by PFASs: A brief literature review

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**Title**

Immunomodulation by PFASs: A brief literature review

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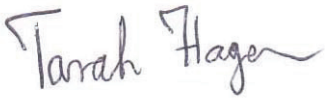

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# Executive Summary

## Introduction

This document is a brief review of the scientific literature that investigates the potential of PFOS and PFOA to modulate the immune system and its functionality. An objective of the review is to determine if there is sufficient robust information to allow modulation of the immune system to be quantitatively considered in human risk assessments for these substances.

The immune system is complex; its functionality relies on many elements to cooperatively operate in concert. The experimental designs and test methods used to examine the function of the immune system are also multifaceted. To assist the reader the basic template underpinning most animal experiments investigating immunomodulation by chemicals is discussed and some of the common test methods explained in an Appendix.

The animal test methods most relevant to understanding if a chemical is likely to adversely perturb immune system function is the *in vivo* production of antibodies in response to vaccination by an antigen and the plaque forming cell assay. Both tests rely on the competency of several components of the immune system to produce antibodies. While high doses, and resulting high serum concentrations, of PFOS or PFOA induce liver hypertrophy, decrease body weight, and decrease the size and cellularity (number and type of cells) of immune organs (thymus and spleen), the tests of immune function (responsiveness) are potentially altered at lower doses and serum concentrations (ie. in the absence of clear systemic toxicity). A decreased responsiveness to antigen in these tests after animals have been treated with the chemical indicates immunosuppression has occurred.

## PFOS animal data

The data indicate PFOS exposure can result in suppression of the primary (initial) antibody response (adaptive immunity) as determined by antigen-specific IgM antibody production to *in vivo* inoculation with T-cell specific antigens in mice. However the doses, and serum concentrations, at which suppression of the antibody response occurs (ie. LOELs) varies widely between studies. Wide dose spacing and studies with the same strain of mice that do not show immunomodulation at much higher PFOS exposures makes identification of a reliable NOEL uncertain. Effects at low doses (low serum concentrations) are reported with gavage dosing while in the same strain of mice dietary exposure resulting in serum concentrations 500 times higher has no effect on the same endpoints. Identification of a NOEL dose in terms of “mg/kg/d” is further exacerbated by different investigators using different ‘daily dose x time’ exposure regimes for exposure periods that are less than the half-life of PFOS in mice. Therefore serum concentration is a better exposure metric than externally applied dose.

Overall the different academic research groups have conducted their work in a scientifically appropriate manner. The weight of evidence indicates PFOS can adversely modulate immune system responsiveness and therefore presents a toxicological hazard for immune effects. However there are marked differences between studies with respect to the exposures necessary to cause such effects, and the quantitative aspects of pivotal studies have not been confirmed in independent investigations.

## PFOS Conclusions

- There are significant uncertainties regarding species sensitivity, strain sensitivity and the influence of route of administration on immune system modulation by PFOS that have yet to be resolved.
- From the studies reviewed a reliable PFOS NOEL or LOEL for possible compromised immune function cannot be determined.
- It is considered inappropriate for potential modulation of the immune system by PFOS to be quantitatively incorporated into human health risk assessments for PFOS exposure at this time.

## PFOA animal data

At high enough doses, for long enough, PFOA causes atrophy and changed cellularity of immune system organs in mice but not in rats. At lower doses, and presumably lower PFOA serum concentrations in mice, PFOA is reported to suppress humoral responses to inoculated antigens. The dose required to do this is about 4 mg/kg/d (LOEL) over a period of 15 days via drinking water (it is uncertain if shorter exposure periods at this dose will also cause immunosuppression). Although the dosing period appears to be shorter than the elimination half-life in mice, and hence the animals may not be at steady state, there is information to indicate pseudo-steady state may be achieved within a few days of the start of dosing; this has yet to be definitively confirmed. Information on PFOA serum concentrations associated with various changes of the immune system is sparse. Nevertheless the available information clearly indicates high serum concentrations, around 70 mg PFOA/L, are required for antibody production suppression.

## PFOA Conclusions

- There is currently insufficient information from animal studies to robustly use the data in quantitative human risk assessment.
- Nevertheless serum PFOA concentrations at which suppression of humoral immune response occurs in animals are very high.
- At such high serum concentrations required for immunomodulation, other toxicological endpoints may be more relevant for risk assessment.

## Epidemiology considerations

There are both positive and negative epidemiology studies showing associations for increasing PFOS and PFOA serum concentrations to compromise antibody production in children and adults. To date there is no compelling evidence for increased incidence of infective disease associated with PFOS or PFOA effects on immune function.

It is difficult to envisage how the available epidemiology information can be used quantitatively in risk assessment.

In June 2016 the US Office of Health Assessment and Translation (OHAT), a division of the National Toxicology Program (NTP), released a draft systematic review of the published literature pertaining to immune system modulation by PFOS and/or PFOA (NTP 2016). The NTP concluded that both PFOA and PFOS are presumed to be immune hazards to humans; this is consistent with the deliberations within this document.

The pivotal outcome of the NTP review is hazard identification and classification, not identification of integrated NO(A)ELs or LO(A)ELs from the literature, or an assessment of immuno-toxicological risk, or risk of health effects that may result from altered immune function. The conclusion by NTP that PFOA and PFOS present an immune hazard to humans means at some level of exposure the function of the immune system may be changed. However the report does not address the issue at what level of exposure is immune function in humans likely to be compromised, as judged either by changed immune parameters or clinical outcome. Hence the NTP review does not assist with determining whether potential immune function modulation by PFOS or PFOA can be quantitatively considered in human risk assessments of these PFASs.

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# Abbreviations

Abbreviations and symbols used in this report are:

## Symbols

| | |
|---|---|
| ↓ | Decrease relative to control |
| ↓↓ | Marked decrease relative to control |
| ↑ | Increase relative to control |
| **Sl↑** | Slight increase relative to control |
| ↔ | No change relative to controls |
| ♂ | Male |
| ♀ | Female |

## Units

| | |
|---|---|
| **kg** | Kilogram |
| **L** | Litre |
| **mL** | Millilitre |
| **µg** | Microgram |
| **ng** | Nanogram |

## Abbreviations

| | |
|---|---|
| **APFO** | Ammonium perfluorooctanoate, PFOA is the anion to this substance |
| **bw** | Body weight |
| **B** | B-cell |
| **Conc** | Concentration |
| **d** | Day |
| **DNP** | Dinitrophenyl-ficoll |
| **DST** | Delayed hypersensitivity test |
| **GD** | Gestation day |
| **Ig** | Immunoglobulin |
| **IL** | Interleukin |
| **lPFOS** | Linear perfluorooctane sulphonate |
| **lAPFO** | Linear ammonium perfluorooctanoate |
| **KO** | Knock out |
| **NK** | Natural killer cell |
| **PFC** | Plaque forming assay |
| **PND** | Postnatal day |
| **Prolif** | Proliferation |
| **SRBC** | Sheep red blood cell |
| **T** | T-cell |
| **TAD** | Total administered dose |
| **TDAR** | T-cell dependent antibody response (eg. with SRBC) |
| **TIAR** | T-cell independent antibody response (eg. with DNP) |
| **wt** | Weight |
| **WT** | Wild type |

# 1 Introduction

The objective of this review is to assess the animal toxicology and epidemiology material for effects of perfluorooctane sulphonate (PFOS) and perfluorooctanoic acid (PFOA) on the immune system, and determine whether there is sufficient information to allow modulation of the immune system to be quantitatively considered in human risk assessments for these substances.

This document is not a review of the immune system or immunotoxicity, the reader is directed to appropriate text books and published reviews for background information on the interconnected intricacies of immune system components and how they effectively cooperate. Nor does the review intend to explore, or speculate on, how changes in production of specific individual elements (eg. an explicit cytokine) by PFOS or PFOA may affect the cascade for functional responsiveness of the system[1]. Rather this review focuses on experimental and epidemiological information for PFOS and PFOA that directly relates to functionality of the immune system, and the exposures necessary for changes in demonstrable effectiveness to occur.

In this review, descriptions of experimental design and the tests used to assess immune system components are kept to a minimum. Nevertheless it is recognised a certain level of knowledge is required to appreciate the information presented. With this in mind, pertinent material is provided in Section 2 and in Appendix A (overview descriptions of common experimental assessment methods) that will assist the reader to understand the animal toxicology and epidemiology investigations described.

# 2 General considerations

The primary role of the immune system of mammals is to protect against foreign bodies and infections.

Its effective function requires cooperative interaction of many components. It is a complex system and within its organisation there are many places where illness, stress, microorganisms, viruses and therapeutic or environmental chemicals may modify its activity and sensitivity (Tryphonas 2001). The immune system is pliable and adaptive, not all changes in the responsiveness of a particular component, or set of components, necessarily results in an adverse health outcome. Depending on the extent of change, an alteration of immune system functionality may or may not increase an organism's risk for adverse health, eg. contraction of a disease. Thus chemical induced changes in immune system responses are not inescapably toxic end points ***per se.*** For these reasons the term immunomodulation, rather than immunotoxicity, is preferred in this review to describe the effects of PFOS and PFOA on the immune system.

It is noted however that many of the tests used to investigate the functionality of the immune system are frequently described as immunotoxicity tests, and the hazard associated with immunomodulation often called an immunotoxic hazard. Without regard to the extent of exposure necessary for the effect, modulation of the immune system by a chemical often results in the chemical being regarded or described as immunotoxic.

Notwithstanding the above, the immune system is recognised as a sensitive target for chemical induced toxicity with many different chemicals being able to modulate its responsiveness. Adverse effects on immune system tissues and compromised function may be manifested in humans as reduced resistance to microbial infection, increased incidence of hypersensitivity (allergic) reactions and autoimmune disorders, and compromised immune surveillance mechanisms responsible for destroying neoplastic cells. These are clinically relevant endpoints used in epidemiology studies. An important unknown is the extent of immune tissue alteration and/or degree of modulation of immune system functionality required to increase host susceptibility to the point clinical effects might occur. Tryphonas (2001) emphasises that the normal immune system has a broad spectrum of reactivity and a great deal of reserve functional capacity. Consequently it is important that changes in immune cell numbers, shifts in cell types, changes in circulating antibodies, or their production are linked to clinically important effects. In addition immune system activity in any given population has genetically related large inter-subject variability. For an individual there is also daily variation in many immune system 'biomarkers'.

1 See DeWitt *et al*. (2012) for a review of possible immune pathways that per- and poly-fluorinated alkylated substances (PFASs) may influence.

Thus there is a myriad of environmental circumstances that may modulate immune system activity and act as confounders in epidemiology studies[2]. All these aspects make epidemiology studies difficult to interpret; demonstrated associations may not be causations, and even if shown to be statistically different to a referent group may not be clinically relevant.

Given the complexity of the immune system it is not surprising that extrapolation of animal immunomodulation effects to humans is challenging and uncertain, particularly if there is lack of dose response and the effects are 'observational' and not 'functional' (see Appendix A).

In keeping with the physiological and biochemical complexity of the immune system, and its various temporal responses to infection, animal tests investigating potential modulation of the immune system by chemicals are also complex. There are many possible endpoints to be considered for assessment.

After treating animals with PFAS, experimental designs investigating immune system status usually use a combination of the following.

- Determination of immune organ status (weight and histology), serum or spleen/thymus immune cell numbers and profiles (ie. organ cellularity).
- Determination of circulating antibodies (IgM and IgG) and cytokines without *in vivo* or after *in vivo* antigen stimulus (eg. injection of Sheep Red Blood Cells, SRBC).
- Immune cell activity measured as ***ex vivo*** activity of isolated Natural Killer (NK) cells or T-cytotoxic cells towards foreign cells.
- *Ex vivo* production of antibodies or cytokines by isolated splenocytes (usually) or thymocytes from animals that have not been, or have been inoculated with antigen (eg. SRBC) at various times towards the end of PFAS treatment and before tissue sampling.
- Release of antibodies and/or cytokines from cultured isolated splenocytes assessed with or without *in vitro* specific stimulation of T-cells or B-cells.

In addition to using different dosage regimes, different species and strains and different PFAS measurement techniques, individual studies used an assortment of immune system assessments (ie. combinations of the above), which further add to the complexity of comparing and interpreting results from different studies.

Although different laboratories may use different tissue preparation techniques and a variety of agents (eg. antigens, cell stimulants) in their assessments of immune system components there is general commonality for the assessment methods used. Hence, instead of providing the assessment details for separate investigations, a brief summary of commonly used assessment tests in animal and epidemiology investigations is compiled in Appendix A.

2 Examples of circumstances that affect immune system activity are age, pregnancy, stress of various forms, hormonal changes, smoking, prescription and non-prescription drugs, heavy metals, and persistent organic pollutants of various types (Tryphonas 2001).

# 3 PFOS

## 3.1 Animal data

Animal investigations have used gavage and dietary administration of PFOS at various daily doses for varying administration times. However combinations of very different dose and administration times can give rise to similar changes in immune endpoints, hence identifying NOELs and LOELs on the basis of daily dose can be misleading. In appreciation of this nuance most studies also report animal exposure as total administered dose (TAD) and provide PFOS serum concentrations at the time the immune system is evaluated. In this review TAD and serum concentrations are used as the external and internal dose metrics respectively.

Serum concentrations are particularly useful since it is well recognised they are directly proportional to dose and effects are proportional to serum concentration[3] (Lau *et al*. 2007, Seacat *et al*. 2002, US EPA 2014, 2016a). Simple one compartment kinetic models have been used to convert an animal NOEL or LOEL serum PFOS concentration to a human dose that will yield the same serum concentration in humans at steady state conditions (DFG 2011, Egeghy and Lorber 2011, Harada *et al*. 2003, MDH 2008, Thompson *et al*. 2010a, US EPA 2016a).

Most studies investigating the immunomodulation properties of PFOS have been undertaken in mice[4].

Unfortunately the toxicokinetics of PFOS in mice is not well characterised. The only study located was by Chang *et al*. (2012). After a single gavage dose[5] of either 1 or 20 mg/kg to CD-1 mice serum concentrations were followed for 85 days; serum elimination was not markedly different between males and females or with dose[6]; the average half-life being 37 days. Since the immunomodulation studies have employed dosing durations of about 7 – 60 days (Table 3.1) it is not expected the animals would have reached steady state conditions for serum PFOS. This further complicates use of external dose (mg/kg/d) as the metric to characterise a NOEL or LOEL.

Individual animal studies with PFOS are summarised in tables in Appendix B. The identified NOELs and LOELs are further consolidated in Tables 3.1 and 3.2.

A number of observations are made from Table 3.1:

- There are principally three academic research groups that have investigated the immunomodulation effects of PFOS.
  - » In the US: Peden-Adams *et al*. (2008), Fair *et al*. (2011), Mollenhauer *et al*. (2011) and Keil *et al*. (2008).
  - » In China: Dong *et al*. (2009, 2011, 2012a, b), Zheng *et al*. (2009, 2011).
  - » In Sweden: Qazi *et al*. (2009a, 2010a, b).
- While each group has observed immunomodulation by PFOS they have done so at very different TAD and PFOS serum concentrations (Tables 3.1 and 3.2).

3 This is due in large part to PFOS being primarily confined to extracellular fluid by virtue of very high and strong protein binding, and the fact it is eliminated slowly in humans. Serum concentrations can be used as a measure of body burden (Butenhoff *et al*. 2004, US EPA 2014, 2016a). Using serum concentrations as a measure of body burden allows serum concentrations associated with NOELs and LOELs in experimental toxicological studies to be compared to human serum concentrations.

4 Mice are a common species for evaluation of immune effects, primarily because they are generally more sensitive than are rats.

5 The PFOS dose in Chang *et al* (2012) was a solution in water with 0.5% Tween 80. Immunomodulation studies in mice have used this vehicle (Peden-Adams *et al*. 2008, Fair *et al*. 2011, Keil *et al*. 2008) or 0.02% Tween 80 (Dong *et al*. 2009, 2011, 2012a, b; Zheng *et al*. 2009, 2011; Guruge *et al*. 2009).

6 Half-life in male and female CD-1 mice was 42.8d and 37.8d respectively after gavage oral dose of 1 mg/kg and 36.4d and 30.4d after 20 mg/kg (Chang *et al*. 2012); average 36.9d. Average volume of distribution (Vd) in mice is 268 mL/kg, consistent with PFOS being distributed primarily to extracellular fluid.

**Table 3.1: Summary of PFOS immunomodulation animal data (serum concentrations mg/L).**

| | Peden-Adams *et al.* 2008 | Fair *et al.* 2011 | Mollen-hauer *et al.* 2011 | Keil *et al.* 2008 | Zheng *et al.* 2009 | Zheng *et al.* 2011 | Dong *et al.* 2009 | Dong *et al.* 2011 | Dong *et al.* 2012a | Dong *et al.* 2012b | Qazi *et al.* 2009a | Qazi *et al.* 2010a | Qazi *et al.* 2010b | Lefebvre *et al.* 2008 | | Guruge *et al.* 2009 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | Mouse | | | | | | Mouse | | | | | Mouse | | Rat | | Mouse |
| Strain | B6C3 F1♂,♀ | B6C3F1 ♀ | | C57BL /6N♀ | | | C57BL/6 ♂ | | | | C57BL/6 ♂ | | B6C3F1 ♂ | SD ♂ & ♀ | | B6C3F1 ♀ |
| Route | Gavage | | | | | | Gavage | | | | | Diet | | Diet | | Gavage |
| Days exposed | 28 | 28 | 28 | GD 1-17 | 7d | 7d | 60 | 60 | 60 | 60 | 10 | 10 | 28 | 28 | | 21d |
| TAD a mg/kg | | | | | | | | | | | | | | ♂ | ♀ | |
| 0 | 0.012± 0.005 | <LOQ 0.001 | | ND | <LOR 0.05 | <LOR 0.05 | 0.048 ± 0.01 | 0.05 ± 0.01 | 0.04 ± 0.01 | 0.04 ± 0.01 | 0.029± 0.01 | | 0.04 ± 0.002 | 0.47 ± 0.27 | 0.95± 0.51 | 0.002 ± 0.0003 |
| 0.005 | 0.018 ± 0.004 | | | | | | | | | | | | | | | |
| 0.05 | 0.092 ± 0.022 | | | | | | | 1.07 ± 0.11 | | 0.58 ± 0.19 | | | | | | |
| 0.1 | 0.13 ±0.015 | <LOQ 0.001 | | | | | | | | | | | | | | 0.189 ± 0.014 |
| 0.5 | 0.67 ± 0.11 ♀ | 1.16 ± 0.09 | | | | | 0.67 ± 0.17 | | | | | | | | | 0.67 ± 0.047 |
| 1 | ND | 2.15 ± 0.55 | ND | ND ~ 1 | | | | 2.36 ± 0.47 | 4.35 ± 0.63 | 4.35 ± 0.63 | | | | | | |
| ~5 | > Calibrat'n | 12.47 ± 0.61 | | | | | 7.13 ± 1.0 | 10.75 ± 0.82 | 8.21 ± 1.15 | 8.21 ± 1.15 | | | 11.6 ± 0.2 | 0.95 ± 0.13 | 1.5 ± 0.23 | |
| 25 | | | | ND ~ 9 | | | 21.64 ± 4.4 | 22.6 ± 2.29 | | 24.5 ± 5.56 | 50.8 ± 2.5 | | | | | |
| –40 | | | | | 110 ± 6.18 | 97.3 ± 7.6 | | | | | | | | 13.45 ±1.48 | 15.4 ±1.56 | |
| 50 | | | | | | | 65.43 ± 11.7 | 51.71 ± 3.8 | 59.7 ± 12.2 | 59.7 ± 12.2 | | | | | | |
| ~100 | | | | ND ~ 50 | | | | | | | 96.7± 5.2 | 125.8 ± 3.9 | | 20.93 ± 2.36 | 31.93 ±3.59 | |
| 125 | | | | | | | 120.7 ± 21.8 | | | 114.2 ± 23.7 | | | | | | |
| ~140 | | | | | 281 ± 16.3 | 250 ± 20.1 | | | | | | | | | | |
| ~200 | | | | | | | | | | | | | | 29.88 ± 3.53 | 43.2± 3.95 | |
| ~260 | | | | | 338 ± 23.9 | | | | | | 340 ±16 | | | | | |

| | Peden-Adams *et al.* 2008 | Fair *et al.* 2011 | Mollenhauer *et al.* 2011 | Keil *et al.* 2008 | Zheng *et al.* 2009 | Zheng *et al.* 2011 | Dong *et al.* 2009 | Dong *et al.* 2011 | Dong *et al.* 2012a | Dong *et al.* 2012b | Qazi *et al.* 2009a | Qazi *et al.* 2010a | Qazi *et al.* 2010b | Lefebvre *et al.* 2008 | Guruge *et al.* 2009 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Critical effect | Decreased PFC after SRBC inoculation at PFOS ≥0.09 mg/L males and female ≥0.67 mg/L. Serum concentration not different between male & female, greater effects in males hence data in table is for male. | Increased release of IL-6 from *ex-vivo* splenocytes (B-cells), other cytokines not affected. No change in organ weight or cellularity. Functional assays not done. Since serum PFOS <LOQ, result uncertain. | TAD 1, 3 & 300 mg/kg. Serum PFOS not measured. Increased serum IL-6 at 1 mg/kg TAD but decreased at 3 mg/kg. Inflammatory responses of peritoneal macrophages variable. Not useful study. | Developmental study. Serum concentrations not measured; values from Lau *et al.* (2007). Decreased NK activity in male, not female, pups at 8 weeks age (↔ 4 weeks), PFC ↔. Wide dose spacing noted. | Decreased PFC & splenocyte T cell proliferation (NK & B cells unaffected), increased liver weight at lowest dose tested = LOEL. NK activity T & B cell proliferation reduced at next dose and above. | Decreased circulating IgM at both doses tested, but increased IgG at lowest dose; IgG unaffected at highest dose. Increase IL-4 from unstimulated *ex-vivo* isolated splenocytes. Very wide dose regime. | Decreased PFC at 7.1 mg/L, NOEL 0.67 mg/L. | Decreased circulating SRBC specific – IgM at ≥10.8 mg/L after *in vivo* inoculation. Increased IL-4 release ex vivo at ≥10.8 mg/L by non-stimulated splenocytes from non-inoculated mice. No change in delayed hypersensitivity test. | Increased splenocyte apoptosis and decreased mitochondrial membrane potential at ≥ 8.21 mg/L. | Decreased body weight and immune organ weights at 24.5 mg/L and above (NOEL 8.21 mg/L). A change in ex vivo release of IL-β from peritoneal macrophages at 8.21 mg/L may be chance finding (no other cytokines altered, no dose response). Cytokine release for splenocytes not increased until 60 mg/L. | Decreased spleen & thymus weight, decreases in all spleen subpopulation phenotypes. Also decreased (ie. loss of) body weight (~25%) at ≥ 340 mg/L serum. | Study examines hepatic immune status. There was decreased cytokines in liver at 125.8 mg/L serum, but T & B-cell function unchanged with stimulus. Only one dose. | Despite decreased body weight gain, no difference from controls for plaque assay, serum IgM & IgG after immunisation, IgM production by isolated splenocytes, cellularity of spleen or thymus. | There was a decrease in circulating IgG1 in males at the lowest exposure tested (tentative LOEL 1 mg/L). However this was not consistent with other antibody measurements and there was no change from controls at any dose for tests designed to assess functionality of the immune system (see text). Therefore lymphocyte apoptosis in thymus of males is chosen as the immunological endpoint. At the LOEL there was also decreased body weight gain and increased liver weight. | Increased response to influenza A infection (increased loss of body weight cf with body weight loss from Inf A alone) at LOEL, increased mortality at next dose. No assessment of antibodies or splenocyte function. Difficult to compare to other studies. |

*ND = Not done*

[a] *For table brevity some TADs in this summary table are an approximation of the actual experimental TAD. Refer Appendix B1 for detailed information.*

NOEL for immune endpoints

LOEL for immune endpoints

Functional endpoint

**Table 3.2: Summary of PFOS NOELs and LOELs by research group.**

| | NOEL | | LOEL | | Dose route/days | Mouse strain/ gender | PFOS analysis |
|---|---|---|---|---|---|---|---|
| | TAD (mg/ kg) | Serum (mg/L) | TAD (mg/ kg) | Serum (mg/L) | | | |
| Peden-Adams *et al*. (2008) | 0.005 | ~ 0.02 | 0.05 | ~0.1 | Gavage 28d | B6C3F1 ♂c | solid phase extraction |
| Dong *et al*. (2009, 2011, 2012a)a | 0.5 - 1 | ~ 2.5 | 5 | ~8.7 b | Gavage 60d | C57BL/6 ♂ | solvent extraction |
| Qazi *et al*. (2010b) | ~ 5 | ~ 12 | ND | ND | Diet 28d | B6C3F1 ♂ | solid phase extraction |

*ND = Not Determined, highest dose was NOEL*
*TAD = Total Administered Dose.*

[a] *Average of 0.67, 2.36 and 4.35 mg/L from three studies (lowest value compromised by dose spacing).*
[b] *Average of 7.15, 10.75 and 8.21 mg/L from three studies.*
[c] *Peden-Adams et al. (2008) used male and female mice, in this publication male mice had greater response to a given TAD (male and female serum concentrations were not significantly different). Hence data in Tables 3.1 and 3.2 are for males.*

» Despite the assessed end points being the same[7] there is respectively an approximate 1000 and 550 fold difference in the TAD and NOEL serum concentrations between Peden-Adams *et al*. (2008) and Qazi *et al*. (2010b). While the mouse strain and gender (male) is the same, the difference might be explained by gavage vs. diet exposure. It is noted dietary exposure to rats also results in a high TAD NOEL and serum NOEL; approximately 40 mg/kg and 13.5 mg/L respectively (Lefebvre *et al*. 2008)[8]. However this might also be due to rats being less sensitive than mice.

The obvious difference between the two studies is gavage versus dietary exposure. Gavage dosing delivers a bolus dose to the stomach and results in higher serum concentration ($C_{max}$) per daily dose and quicker time ($T_{max}$) to $C_{max}$ than does an equivalent daily dose from the diet. If effects on the immune system are related to peak *in vivo* serum PFOS concentrations then the difference between the NOELs after gavage versus dietary exposure might be related to different PFOS kinetics after gavage or dietary exposure, and/or timing after gavage dose that animals are killed and blood is obtained[9].

» Serum PFOS concentrations achieved by the Peden-Adams group (Peden-Adams *et al*. 2008, Fair *et al*. 2011) and the Dong research group (Dong *et al*. 2009, 2011) appear to be approximately equivalent for a given TAD[10]. Although both groups measured the ability of animals to mount specific antibody responses[11] to the same antigen (SRBC), the NOELs from each are very different. The NOEL from Dong *et al*. (2011) is 200x higher than that from Peden-Adams *et al*. (2008) based on TAD, and approximately 120x higher based on serum PFOS concentration (Tables 3.1 and 3.2). Both research groups dosed male mice by gavage but used different strains (B6C3F1 vs. C57BL/6) and slightly different dose vehicles (0.5% Tween 80 vs. 0.02% Tween 80), although the latter is not expected to account for the difference since PFOS is completely absorbed[12] from the gastrointestinal tract (Chang *et al*. 2012).

7 For Peden-Adams *et al*. (2008) the NOEL relies on decreased PFC after inoculation with SRBC (it is noted circulating SRBC specific IgM after inoculation was not measured, nor in plaque assay). In Qazi *et al*. (2010b) the critical endpoints are no change in PFC assay, no change in circulating SRBC specific IgM or TNP-LPS specific IgM after inoculation with SRBC or TNP-LPS antigen. Arguably there are more assays in Qazi *et al*. (2010b) that are relevant for immune system functionality than in Peden-Adams *et al*. (2008); 3 vs.1.

8 The studies by Lefebvre *et al*. (2008) were conducted in the laboratories of the Food Directorate, Health Products and Food Branch, Health Canada.

9 Peden-Adams *et al*. (2008) and Qazi *et al*. (2010b) both inoculated PFOS treated male mice with intraperitoneal SRBC 5 days prior to sacrifice, Peden-Adams *et al*. (2008) do not indicate the time after the last gavage PFOS dose that animals were killed for evaluation.

10 Although not reported in Peden-Adams *et al*. (2008) the serum concentration at a TAD of 1 mg/kg in experiments from this research group is 2.2 mg/L (Fair *et al*. 2011) and is similar to the serum concentrations (2.4 mg/L) of Dong *et al*. (2011) at this TAD. At a TAD of 0.5 mg/kg, Peden-Adams *et al*. (2008) report a serum PFOS concentration of 0.67 mg/L, the same as reported by Dong *et al*. (2009).

11 Although Dong *et al*. (2011) did not measure plaque forming cells (PFC) as in Peden-Adams *et al*. (2008), they did assess the functionality of the immune system after PFOS administration by measuring circulating SRBC specific-IgM after inoculating animals with SRBC, and also by assessing a delayed hypersensitivity response mediated by SRBC specific-IgE.

12 Although PFOS is completely absorbed from the gastrointestinal tract, conceivably different dose vehicles could influence the maximum serum concentration ($C_{max}$ ) from each dose and time to $C_{max}$ ($T_{max}$).

» Guruge *et al*. (2009) found increased susceptibility of mice to influenza A after gavage PFOS treatment for 21 days. No NOAEL was identified but the LOAEL was at a TAD of 0.1 mg/kg and serum concentration of 0.19 mg/L. This is the only study available that has used an infectivity model to assess the effects of PFOS on immune responses to infection, as such it is pertinent to have the result verified by other investigators.

Nevertheless infectivity models correlate strongly with other immune function tests and are highly predictive of chemical-induced immunosuppression (Luster *et al*. 1992, 1993).

## 3.2 Discussion and conclusions on PFOS animal data

High PFOS doses resulting in toxicity (body weight loss and hepatomegaly) in mice also caused splenic and thymic atrophy and immunosuppression, including depressed natural killer cell activity, lymphocyte proliferation, and T-cell dependent antibody response. However, such immunomodulation findings cannot reliably be attributed to a direct immunotoxic effect due to the concomitant general toxicity and stress.

The data indicate PFOS exposure can result in suppression of the primary (initial) antibody response (adaptive immunity) without marked general toxicity as determined by antigen-specific IgM antibody production to *in vivo* inoculation with T-cell specific antigens (SRBC) in mice (Peden-Adams *et al*. 2008, Dong *et al*. 2009, 2011; Zheng *et al*. 2009, Qazi *et al*. 2010b). However the doses, and serum concentration, at which suppression of the antibody response occurs (ie. LOELs) varies widely between studies. Wide dose spacing and studies with the same mice that do not show immunomodulation at much higher PFOS exposures makes identification of a reliable NOEL uncertain.

Differences between studies for the same functional endpoint (antibody production to a common antigen, SRBC) may be related to strain or dose regime differences, but to date these have not been resolved. The lowest NOEL (as either TAD [0.005 mg/kg] or serum concentration [0.018 mg/L]) for an effect by PFOS on a functional endpoint of the immune system is reported by Peden-Adams *et al*. (2008). It relies solely on a decrease in the number of plaque forming cells (PFC) in the spleen of PFOS treated animals inoculated with SRBC; antibodies specific for SRBC were not measured either in serum or in the plaque assay. It is also noted the research group have not repeated the study to confirm the effect occurs at the very low PFOS doses and serum levels in Peden-Adams *et al*. (2008). In contrast Qazi *et al*. (2010b), at 1000 fold higher TAD and 550 times higher PFOS serum concentration could not replicate the Peden-Adams *et al*. (2008) findings, albeit using dietary exposure but more measurements of the same immune response. The NOELs from other researchers (Dong *et al*. 2009, 2011) for impacts on functional endpoints fall between Peden-Adams *et al*. (2008) and Qazi *et al*. (2010b). It is also noted the dietary NOEL for functional endpoints in rats is similar to that in mice (Lefebvre *et al*. 2009, Qazi *et al*. 2010b).

Increased influenza A effects in PFOS treated mice suggests impacts on the immune system (Guruge *et al*. 2009). However this study only used two PFOS doses and did not identify a NOAEL and the results have not been confirmed by other investigators. In addition the study did not investigate whether there were changes in antibody response to the virus in PFOS treated animals compared to controls.

Overall the different academic research groups have conducted their work in a scientifically appropriate manner. The weight of evidence indicates PFOS can adversely modulate immune system responsiveness and therefore presents a toxicological hazard for immune effects. However there are marked differences between studies with respect to the exposures necessary to cause such effects, and the quantitative aspects of pivotal studies have not been confirmed in independent investigations.

**Conclusions**

- There are significant uncertainties regarding species sensitivity, strain sensitivity and influence of route of administration on immune system modulation in experimental animals by PFOS that have yet to be resolved.
- From the studies reviewed a reliable PFOS NOEL for possible compromised immune function cannot be determined.
- It is considered inappropriate for potential modulation of the immune system by PFOS to be quantitatively incorporated into human health risk assessments for PFOS exposure at this time.

# 4 PFOA

## 4.1 Animal data

Two principal academic groups have investigated the immunomodulation effects of PFOA.

- From the US (DeWitt *et al*. 2008, 2009c, 2016; Hu *et al*. 2010, 2012).
- From Sweden (Yang *et al*. 2000, 2001, 2002a,b; 2006, 2011).

Table 4.1 summarises the dose response information from these groups, and others, on immune system functionality as measured by production of antigen-specific IgM after inoculating PFOA treated animals (usually mice) with antigen (eg. SRBC), ie. measuring TDAR or TIAR.

The studies by Yang *et al*. (2000, 2001, 2002a) are not included in Table 4.1 as they have primarily utilised observational tests of the immune system. In these studies decreases in thymus and spleen weight, and their cellularity, have been correlated with decreased body weight and increased liver weight and with peroxisome proliferation. Comparisons with other PPARα agonists are also made[13]. These investigations have not measured the effects of PFOA on the function or responsiveness of the immune system. While they contribute towards understanding the immunomodulatory mode of action of PFOA[14] they do not inform on useful aspects of the dose response or identification of NOELs for immune endpoints of interest.

Yang *et al*. (2002b) exposed male C57BL/6 mice to a single concentration (0.02%) of PFOA in the diet for 16 days. TDAR was measured after inoculating PFOA treated mice with horse red blood cells (HRBC) intravenously on day 10; serum levels of HRBC-specific IgM and IgG in response to the immunisation were significantly decreased. Lymphocyte proliferation by isolated splenocytes from PFOA treated animals (no antigen inoculation) in response to Con A or LPS stimulation was also significantly supressed as was PFC. While this study demonstrates PFOA at a high dose (approximately 30 mg/kg/d) can functionally inhibit adaptive immune response, it is of limited use because only one dose was used and hence a NOEL or robust LOEL cannot be determined from the study.

In contrast to the observational/mode of action investigations of Yang *et al*. (2000, 2001, 2002a, b) DeWitt *et al*. (2008, 2009c, 2016) have employed functional assays (TDAR and TIAR) to identify NOELs and LOELs for PFOA administered at various doses in drinking water over 10 – 15 days. These latter studies provide relevant dose response information.

All the studies investigating PFOA induced immunomodulation are short term exposures that are less than, or comparable to the 22 and 16 day PFOA serum half-life in male and female mice (Lou *et al*. 2009). Based on half-life considerations it would be anticipated the studies would not achieve serum PFOA steady state conditions, or likely come close to steady state. However from the data of Lau *et al*. (2006) and Lou *et al*. (2009) it is apparent steady state PFOA serum concentrations are reached much quicker (within 7 days) after repeat dosing than implied by the half-life. Simulation modelling of daily repeat dose of 20 mg/kg/d, yielding a serum concentration approximately 130 – 180 mg PFOA/L, using a saturable renal resorption model indicated pseudo-steady state is reached very rapidly, two days after the first dosing (Lou *et al*. 2009). Therefore the PFOA repeat dose immunomodulation studies in Table 4.1 and Appendix B may be at steady state at the time tests for immune system status are conducted on the animals, at least for the high doses.

13 The peroxisome proliferators investigated were PFOA, di(2-ethylhexyl)phthalate (DEHP), Wy-14 643 and nafenopin. Cellularity changes included decreases in CD4+ and CD8+ cells, altered T-cell populations, in spleen and thymus.

14 At the doses used to investigate splenic and thymus cellularity in mice (0.02% in the diet, approximately 30 mg/kg bw/day) there was overt toxicity, as indicated by marked body weight loss (~15 – 30%) (Yang *et al*. 2000, 2001, 2002a). It was hypothesised the observed immune suppressive effects could be by an indirect pathway such as being secondary to toxicity and stress rather than a specific effect by PFOA on the immune system. The hypothesis was subsequently investigated by DeWitt el al. (2009c) who demonstrated the immune suppression effects of PFOA were independent of systemic toxicity or stress. In addition, since in 'stop' experiments thymus and spleen atrophy and cellularity recovery was faster (within 10 days of PFOA withdrawal) than peroxisome proliferation and liver weight, it thus appeared that peroxisome proliferation per se may not be entirely responsible for the immunological suppression effects (Yang *et al*. 2001).The conclusion by Loveless *et al*. (2008) that decreases in immune organs and TDAR in mice only occurred in the presence of systemic toxicity is a direct consequence of wide dose spacing in which the LOEL is over estimated and the NOEL under estimated (see Table 4.1).

**Table 4.1: Summary of effects of PFOA on immune system function (TDAR) in animals.**

| | DeWitt *et al.* 2008 | | De Witt *et al.* 2016 | DeWitt *et al.* 2009c. | Love-less *et al.* 2008 | |
|---|---|---|---|---|---|---|
| **Species** | Mouse | | | | Rat | Mouse |
| **Strain** | C57BL/6 ♀ | | | | CD ♂ | CD-1 ♂ |
| **Route** | Drinking water | | | | Gavage | |
| **Days exposed** | 15 | | 15 | 10 | 29 | |
| **mg/kg/d** | serum (mg/L) a | | | | | |
| **0** | | | | | | |
| **0.3** | | | | | ↔ | ↔ |
| **~1** | ↔ | ND b | ↔ | | ↔ | ↔ |
| **1.88** | ↔ | ND b | ↓TIAR c | | | |
| **3.75** | ↓ | 74.9 ± 2.7 a | ↓TIAR c | | | |
| **7.5** | ↓ | 87.2 ± 3.3 a | ↔ TDAR ↓TIAR c | ↔ | | |
| **10** | | | | | ↔ | ↓ |
| **15** | ↓ | 128.1 ± 6.8 a | | ↓ | | |
| **30** | ↓ | 162.6 ± 8.4 a | ↓TDAR & TIAR | | ↔ | ↓ |

*(An empty cell in the table signifies there are no data for that location)*
*NOEL for TDAR or TIAR*
*LOEL for TDAR or TIAR*

↓ **:** *Decrease relative to control.* ↔**:** *No change relative to controls.*
a *PFOA concentration in aliquots of serum collected for measurement of IgM titre 1 day post dosing.*
b *Serum PFOA measurement not done.*
c *The decrease TIAR relative to 0 mg/kg PFOA was 10.3%, 9.4% and 10.7% for 1.88, 3.75 and 7.5 mg/kg/d respectively*[15]*. That is, there was no dose response.*

| | **Pivotal endpoints** (see Appendix B for study descriptions) |
|---|---|
| DeWitt *et al* 2008 | NOEL for TDAR (SRBC-specific IgM) = 1.88 mg/kg/d; BMDL1SD =1.75 mg/kg/d. Serum PFOA at these doses not measured. LOEL = 3.75 mg/kg/d (Serum PFOA 75 ± 2.7 mg/L). |
| DeWitt *et al*. 2016 | Investigation of mode of action for T- & B-cell effects & role of PPARα.<br>NOEL = 0.94 mg/kg/d for TIAR in C57BL/6N mice but no dose response at higher doses; given the decrease at each dose above NOEL is only ~10%, confirmation is ideally required before using this study for risk assessment.<br>NOEL = 7.5 mg/kg/d for TDAR (IgM) in WT (C57BL/6-Tac). Serum PFOA not done. |
| DeWitt *et al*. 2009c | Investigation of involvement of stress & corticosterone (does not influence TDAR effects).<br>Decreased body wt & TDAR at NOEL (7.5 mg/kg/d). Serum PFOA not done. |
| Loveless *et al*. 2008 | No immune effects in rats even though marked ↓ body weight gains. In mice immune effects only at toxic doses (NOEL = 1mg/kg/d for TDAR IgM). Authors concluded immune effects secondary to systemic toxicity & stress (↑ corticosterone) but note the wide dose spacing between the NOEL and LOEL. |

As with PFOS, the short and variable length exposures make identification of a reliable NOEL for immune effects problematic. Furthermore, unlike similar studies with PFOS, investigations with PFOA do not report total administered doses (TAD) and only one has measured serum PFOA after dosing (DeWitt *et al*. 2008).

Unfortunately the measured serum PFOA concentrations in DeWitt *et al*. (2008) are only available for the LOEL, and not the NOEL identified by the study. Nevertheless it appears that NOELs and LOELs for suppression of functional aspects of the immune system are associated with very high PFOA serum concentrations (Table 4.2). Interestingly

15 Doses in DeWitt *et al* (2016) were calculated according to water bottle weights measured twice per week with 4 animals per cage.

PFOA serum concentrations in DeWitt *et al*. (2008) at the lower doses, ie. serum concentrations ≤ 90 mg/L, imply a PFOA serum half-life of about 15 days which is the same as that reported by Lou *et al*. (2009) for female CD1 mice [16].

**Table 4.2: Serum PFOA concentrations from De Witt *et al*. (2008).**

| Dose (mg/kg/d) in drinking water for 15 days | PFOA serum concentration (mg/L) a | |
|---|---|---|
| | 1 day post dosing | 15 days post dosing |
| 0 | 0.054 ± 0.002 | 0.156 ± 0.067 b |
| 0.94 | ND c | ND |
| 1.88 | ND | ND |
| 3.75 | 74.9 ± 2.7 | 35.3 ± 1.6 |
| 7.5 | 87.2 ± 3.3 | 42.7 ± 1.7 |
| 15 | 128.1 ± 6.8 | 50.0 ± 1.5 |
| 30 | 162.6 ± 8.4 | 52.7 ± 3.2 |

*a Values rounded from DeWitt et al. (2008).*
*b Unknown why controls had higher serum PFOA at 15 days post dosing.*
*c ND = Not Done.*
*NOEL, LOEL. See Table 4.1 and Appendix B.*

## 4.2 Discussion and conclusions on PFOA animal data

The reported serum half-life of 16 – 22 days in mice suggests immunomodulation studies with PFOA in which exposure has been for about 10 – 30 days are unlikely to be at steady state serum concentrations. However data outside of the immunomodulation studies indicate pseudo steady state may be achieved within 2 – 7 days of the start of daily repeat dosing. There is uncertainty regarding this tentative conclusion because frequent PFOA serum measurements have not been reported within the dosing periods employed to investigate PFOA induced immunomodulation.

At high enough doses, for long enough, PFOA causes atrophy and changed cellularity of immune system organs in mice but not in rats. At lower doses, and presumably lower PFOA serum concentrations in mice, PFOA is reported to suppress humoral responses to antigens. This is principally measured as decreased amounts of circulating antigen-specific antibody after animal inoculation (measuring IgM), or subsequent challenge (measuring IgG), with the test antigen (usually SRBC, ie. T- cell dependent [TDAR]). The suppression of TDAR has been observed in several studies and a NOEL of 1.88 mg/kg/d and LOEL of 3.75 mg/kg/d identified for PFOA administered in drinking water over 15 days (DeWitt *et al*. 2008); on modelling the TDAR dose response data the authors estimated a bench mark dose ($BMD_{1SD}$) of 1.75 mg/kg/d.

More recently DeWitt *et al*. (2016), in studies designed to investigate mechanistic aspects of PFOA immunomodulation, have observed a decrease in TIAR to DNP antigen (this chemical is associated with delayed hypersensitisation). The NOEL was 0.94 mg/kg/d and the decrease only 10% at the LOEL of 1.88 mg/kg/d with the magnitude of the effect the same at higher doses. The lack of dose response to DNP and absence of serum PFOA measurements in this study raises an issue regarding the reproducibility of the TIAR result(s), their biological significance, and limits the usefulness of the data.

If the dose regime used by the DeWitt research group (ie. 15 days in drinking water) results in steady state PFOA serum concentrations, as discussed above, then the daily NOEL would be anticipated to be valid for longer dosing periods. This is however unknown. It is noted there is little concordance between NOELs and LOELs from different studies when exposure is expressed as total administered dose[17].

16 It is noted that at PFOA serum concentrations of approximately ≤ 100 mg/L, elimination of PFOA from mouse serum complies well with linear first order kinetics (ie. a simple one compartment model) (Lou *et al*. 2009).
17 TAD is calculated as the product of daily dose and number of days.

Information on PFOA serum concentrations associated with various changes of the immune system is sparse. Nevertheless the available information clearly indicates high serum concentrations are required.

**Conclusions**

While immunomodulation by PFOA is potentially an end point of concern it is suggested there is currently insufficient information from animal studies to robustly use the data in quantitative human risk assessment. The studies have a number of limitations which include uncertainty regarding steady state, dose spacing, incomplete or no serum PFOA measurements, and for some endpoints for which there are differences from controls there is no dose response. Furthermore it is noted the kinetics of PFOA at the serum concentrations achieved in mice, and other experimental animals, is complex and difficult to model (eg. Anderson *et al*. 2006, Rodriguez *et al*. 2009, Lou *et al*. 2009, Loccisano *et al*. 2011, US EPA 2016b).

Nevertheless it is clear from the available animal evidence serum PFOA concentrations at which suppression of humoral immune response occurs are very high. It is also noted that at such serum concentrations endpoints other than immunomodulation may be more relevant, however those considerations are beyond the scope of this review.

# 5 Developmental immunomodulation

## 5.1 PFOS

Keil *et al*. (2008) gavaged C57BL/6N mice on gestation days 1 – 17 with lPFOS (0.1, 1 and 5 mg/kg/d). Serum concentrations were not measured but extrapolation of data from Lau *et al*. (2007) suggests respective concentrations of 1, 9 and 50 mg/L would be anticipated in dams at time of parturition.

- There were no effects on immunomodulation end points (NK cell activity and PFC) in male or female pups when 4 weeks old with ≤ 1 mg/kg/d.
- At 8 weeks of age:
  - » Male pups had decreased NK cell activity at 1 mg/kg and NK activity was decreased in both gender offspring at 5 mg/kg.
  - » In male pups, not females, plaque cell formation (PFC), a measure of SRBC-specific IgM, was decreased at 5 mg/kg/d. At this dose there were also changes in splenocyte sub-populations in male offspring but not females.

In this study the PFOS developmental immunomodulation NOEL for NK cell activity was 0.1 mg/kg/d, and for specific IgM production and splenocyte cell changes the NOEL was 1 mg/kg/d. It is noted the wide dose spacing confers uncertainty on the NOEL for NK activity, additional studies are required to confirm the NK cell effects and the apparent higher sensitivity of male pups. The study is described in greater detail in Appendix B.

## 5.2 PFOA

To investigate possible developmental immunomodulation by PFOA, Hu *et al*. (2010) exposed C57BL/6 mice on gestation days 6 – 17 to 0.5 and 1 mg/kg/d via drinking water (see Appendix B). In common with Keil *et al*. (2008) was assessment of SRBC- specific IgM, albeit by different methods. Hu *et al*. (2010) measured serum SRBC-specific IgM and IgG after inoculating 5 week old female pups with SRBC (ie. immediate and late TDAR), whereas Keil *et al*. (2008) measured specific IgM indirectly via the PFC assay. Hu *et al*. (2010) found no difference from controls in TDAR response.

The NOEL for developmental immunomodulatory effects by PFOA is 1 mg/kg/d. In pilot studies Hu *et al*. (2010) found pups did not survive maternal doses >1mg/kg/d[18]. This is consistent with the traditional developmental data reported by Lau *et al*. (2006); for neonatal mortality (determined by survival to weaning) these researchers estimated a $BMD_5$ and $BMDL_5$ of 2.84 mg/kg/d and 1.09 mg/kg/d respectively[19]. It therefore appears that developmental immunomodulation effects of PFOA coincide with significant toxicity to the foetus/neonate.

18 At a dose of 5 mg/kg/d in drinking water to pregnant mice 75% of pups did not survive (Hu *et al*. 2010).

19 In Lau *et al*. (2006) PFOA was given daily by gavage to pregnant mice on GD 1 – 17 at 1, 3, 5, 10, 20 or 40 mg/kg/d. After 7d at 10 mg/kg, PFOA in serum was 178 ± 0.0 19 mg/L and after 17 days 171 ± 15 mg/L.

In Hu *et al*. (2010) serum concentrations were measured in pups at different ages but not in dams at parturition (Table 5.1). Compared with adult mice where half-life is reported to be 22 and 16 days in males and females (Lou *et al*. 2009), the data in Table 5.1 suggests more rapid depuration of PFOA from young animals, growth dilution would also contribute. Although serum PFOA was not measured in dams the concentrations measured in pups 20 days after birth indicate concentrations in dams at the NOEL would have been in excess of 3.5 mg/L.

**Table 5.1: Serum PFOA in offspring of dams dosed GD 6 -17 in drinking water**

| Pup gender | Age (days) | Serum PFOA (mg/L) a | |
|---|---|---|---|
| | | 0.5 mg/kg/d | 1 mg/kg/d |
| Male | 20 | 1.56 | 3.41 |
| Female | 48 | 0.12 | 0.18 |
| Female | 63 | ~0.02 | ~0.055 |

*a Data are mean serum concentrations, some values are approximate as they are read from a graph in Hu et al. (2010).*

In an abstract Yang *et al*. (2011) suggested PFOA (5 mg/kg/d) orally administered to C57BL/6J mice during gestation and lactation may have the effect on the immune system of both dams and offspring (decreased thymic cellularity in dams and increased T-cell numbers in PND21 offspring). No information is provided regarding maternal or pup toxicity and serum PFOA is not reported. It is implied no effects were observed in pups at 1 mg/kg/d.

In another study Hu *et al*. (2012) explored the hypothesis that developmental exposure to PFOA may induce immunotoxicity similar to that observed in subsets of patients with neurodevelopmental disorders. To test the hypothesis C57BL/6N mice were exposed via gavage to 0.02, 0.2 and 2 mg/kg/d for an average of 13 days prior to conception, through gestation and lactation until offspring were weaned. When male and female offspring reached adulthood (approximately 6 weeks of age) spleens were removed and assessed for IL-10 production and immunophenotyping of splenocytes. In addition serum autoantibodies[20] and brain endpoints[21] were measured. Serum PFOA concentrations were not measured in dams or offspring. Terminal body weights of dams and pregnancy indices were not changed at any dose but litter weights at the top dose were significantly lower than other groups through to PND21 when offspring were sacrificed. The authors concluded that at developmental exposure levels below 2 mg/kg, no definitive changes indicative of the types of immunopathologies observed in neurodevelopmental disorders exist, but at 2 mg/kg certain cells[22] of the immune system can be altered by developmental exposure to PFOA. Since functional aspects of the immune system in offspring were not evaluated in this study it is only speculative to suggest adverse immune function may arise as a result of the changed splenocyte profile. With the wide dose spacing and type of investigation tests we consider this study is not conducive for NOEL determination.

## 5.3 Conclusions

In offspring of mice, high doses of PFOS (5 mg/kg/d by gavage expected to give a dam serum concentration around 50 mg/L) and PFOA (1 mg/kg/d via drinking water generating a pup serum concentration >> 3.4 mg/L at birth) during pregnancy results in a decrease in production of specific IgM by splenocytes in response to SRBC inoculation (PFC assay). That is a decrease in the ability of offspring to mount a humoral immune response to a T-cell dependent antigen. The maternal NOELs for this effect are for:

- PFOS 1 mg/kg/d.
- PFOA 1 mg/kg/d.

Decreases in NK cell activity by PFOS at lower doses are uncertain as it occurred only in male mice when they were 8 weeks old and not at 4 weeks of age, and in female offspring at the high dose at 8 weeks of age.

20 Autoantibodies looked for in Hu *et al*. (2012) were antidsDNA, anti-ssDNA, and anti-myelin basic protein (anti-MBP).
21 T cell infiltration and myelin basic protein levels in cerebella.
22 The mean percentage of splenic CD4+CD25+Foxp3+ T cells (Tregs) within CD4+ cells was decreased in male and female offspring.

End of pregnancy maternal serum concentration at the above NOELs were not measured in the studies but:

- By extrapolation from another study with pregnant mice it is presumed to be around 9 mg/L for PFOS.
- From serum levels in 20 day old offspring it is expected to be much greater than 3.4 mg/L for PFOA.

Since there is only one developmental immunomodulation study for each of PFOS and PFOA the above doses and serum concentrations need to ideally be confirmed by additional studies before use in quantitative risk assessment.

The above NOELs for developmental immunomodulation are placed in context by comparison with findings from developmental studies in which other endpoints have been measured. For example, the $BMD_5$ and $BMDL_5$ for neonatal mortality when PFOA is administered to mice during pregnancy are 2.84 mg/kg/d and 1.09 mg/kg/d respectively. This suggests toxicity other than immunomodulation may be the critical endpoints of concern for risk assessment.

# 6 Epidemiology data

The epidemiology literature examining relationships between PFAS exposure and immunomodulating effects in humans has been reviewed by Corsini *et al*. (2014), DeWitt (2015), Chang *et al*. (2016) and NTP (2016). Common to the reviews is concern that prenatal and early childhood exposure to environmental factors may cause changes in immune system development such that susceptible people are laid open to greater risk for diseases later in life. Chang *et al*. (2016) is a systematic review which concluded the available epidemiologic evidence is insufficient to reach a conclusion about a causal relationship between exposure to PFOA and PFOS and any immune related health condition in humans. NTP (2016) is also a systematic review that concluded both PFOS and PFOA should be presumed to be an immune hazard to humans; this is based primarily on evidence these chemicals suppress the antibody response in animals (Sections 3 and 4). The NTP (2016) review is silent on possible effective serum concentrations for immunomodulation in humans since it was conducted for the purpose of hazard identification and not dose response assessment for determining a NO(A)EL (Appendix C).

The possibility that PFASs attenuate the positive benefits of vaccination in children has received particular attention. Recent studies have suggested an association between PFAS body burden and lower vaccine protection (Grandjean *et al*. 2012, Granum *et al*. 2013, Looker *et al*. 2014, Kielsen *et al*. 2016). Given the weight of evidence to suggest decreased humoral response in animals with increasing serum PFOS and PFOA the concept has biological plausibility. Grandjean *et al*. (2012) and Granum *et al*. (2013) are prospective birth cohort studies for populations in the Faroe Islands and Norway respectively. Looker *et al*. (2014) and Kielsen *et al*. (2016) are cross-sectional studies. All these studies have found a negative association between PFAS serum concentrations and antibody response to various vaccines. However the effect is usually weak and not consistent for all vaccines.

In addition some studies, while observing decreased antibody titre, have not found significant increases in incidence of human disease or associations of higher serum PFAS with infectious disease (Leonard *et al*. 2008, Fei *et al*, 2010a, Granum *et al*. 2013, Okada *et al*. 2014, Looker *et al*. 2014, Ashley-Martin *et al*. 2015). But one study has found an association between high PFAS and disease incidence (Dong *et al*. 2013).

The Grandjean *et al*. (2012) study is arguably the most cited investigation for suppressed vaccination outcome in children. It is a prospective study of a birth cohort of 587 children from the National Hospital in the Faroe Islands. The exposure of this cohort to PFASs is primarily via their high consumption of seafood. Prenatal exposure was assessed with PFAS measurement in maternal serum during the third trimester of pregnancy and in children's blood when 5 years old. Maternal serum concentrations were for PFOS (0.023 – 0.033 mg/L) and PFOA (0.003 – 0.004 mg/L) and well within normal background concentrations. The investigators measured tetanus and diphtheria antibodies in children aged 5 and 7 years (not all children participated at both times), before and after immunisation with the diphtheria and tetanus booster vaccine. Higher maternal prenatal serum PFOA and PFOS were not associated with a significant difference in response to the tetanus vaccine in children[23] but there was a negative association with diphtheria vaccine response with some children being at a non-protective antibody level. A 2-fold increase in PFOS and PFOA concentrations at age 5 years was associated with odds ratios of 2.38 (95% CI, 0.89 to 6.35) and 4.20 (95% CI, 1.54 to 11.44) for falling below a clinically protective level for tetanus and diphtheria antibodies, respectively,

23 There was however a significant positive association between higher maternal PFOS and higher anti-tetanus antibody titre at age 7 years when adjustments were made for changes due to age.

at age 7 years. Chang *et al*. (2016) describes several issues associated with this study; overall they consider the variability in findings, particularly between vaccines, and the several outcome measurements[24] make the results difficult to interpret. Grandjean and Budtz-Jørgensen (2013) used the data to calculate a $BMDL_5$[25] of 0.0013 mg/L for PFOS and 0.0003 mg/L for PFOA; these are very low serum concentrations.

In a wide ranging review of the epidemiology literature, Dietert (2014) concluded air pollution, aluminium, antibiotics, arsenic, bisphenol A, ethanol, lead, maternal smoking and environmental tobacco smoke, paracetamol, pesticides, polychlorinated biphenyls, as well as PFASs were risk factors for developmental immunotoxicity. Similarly, Gascon *et al*. (2013) after reviewing 41 studies for prenatal exposure to DDE, PCBs and dioxins for the risk of respiratory infections in childhood concluded there was evidence, albeit limited, for early-life exposure to persistent organic pollutants (POPs) adversely influencing immune system development.

It is therefore possible that variables other than PFOS or PFOA may have contributed to lower vaccine antibody titre in the Faroe Island study, particularly if there is a common exposure pathway such as dietary fish or whale meat. Indeed for the Faroe Island cohort a number of environmental pollutants in the blood of mothers or children (PCBs, mercury as well as various PFASs) have been associated with altered levels of various antibodies[26] in children (Grandjean *et al*. 2010, Heilmann *et al*. 2006, 2010; Osuna *et al*. 2014). Some of the associations are much stronger than for the PFASs. Recently Mogensen *et al*. (2015) have attempted to model the association of PFOS, PFOA and PFHxS concentrations in 7 year olds with diphtheria and tetanus antibodies and concluded while each was individually associated with a decrease in antibody concentration it was not possible to attribute causality.

## 6.1 Discussion and conclusions

Overall, the epidemiology data are not sufficient to establish a causal effect between PFOA or PFOS exposure (serum concentrations) with clinically relevant impaired serological vaccine response. Some of the positive associations require replication in independent studies.

A common issue with the epidemiology studies is dislocation of the exposure and effect measures. For example, often the antibody response to vaccination is assessed some months or years after the vaccination event and PFAS exposure assessment may be some years prior to vaccination. The disjointed exposure-effect nexus hampers dose response evaluation and effectively relegates most epidemiology studies to being hazard assessments. Consequently epidemiology studies investigating immunological effects of PFASs have not been used by regulatory agencies to derive toxicity reference values (ATSDR 2015, CoT 2006a,b, CoT 2009, Danish EPA 2015, EFSA 2008, German DWA 2006, Maine DHHS 2014, MDH 2008, US EPA 2016a,b).

Statistical analysis within PFAS epidemiology studies is often not with a 'control' population but between measurement strata of the study population whereby associations are found when the lowest PFAS exposure (eg. the lowest quartile) is compared with the highest PFAS exposure (eg. highest quartile). But the different strata are usually over a low and narrow serum concentration range, eg. 0.002 – 0.05 mg/L for PFOS, which is within background serum concentrations. No doubt this contributes to the weak associations and inconsistency between studies. As does different assessment methodologies used for assessing immunomodulation. Furthermore, the statistical analysis in many studies is not easily understood with respect to how a particular PFAS was dissected out for a positive association but other co-exposure PFASs show no association. It is also noted that many of the associations are weak, the effects small and of questionable clinical significance.

There are also many chemicals known to have immunomodulating effects, and which for some there may be co-exposure with PFASs but have not been addressed in epidemiology studies for PFASs.

24 Tested associations were for concentrations (interquartile range) of 5 PFASs (PFOS, PFOA, PFHxS, PFNA, PFDA) in prenatal or childhood (at 5 years) serum with vaccine antibody levels for two different vaccines at 5 and 7 years old, with and without booster vaccine.

25 The BMDL5 calculated by Grandjean and Budtz-Jørgensen (2013) is the lower bound confidence limit for a serum concentration of PFOS or PFOA in a child 5 years old that may reduce antibody levels when 7 years old by 5%. The health implications of this BMDL5 are obscure to this reviewer.

26 The various antibodies measured include neural (neurofilaments, cholineacetyltransferase, astrocyte glial fibrillary acidic protein and myelin basic protein) and non-neural (actin, desmin, and keratin) autoantibodies (Osuna *et al*. 2014); total immunoglobulin E (IgE) and grass-specific IgE (Grandjean *et al*. 2010); tetanus and diphtheria (Heilmann *et al*. 2006); diphtheria (Heilmann *et al*. 2010).

### Conclusion

The epidemiology information suggests PFOS and PFOA may present an immune hazard to humans but the exposure levels (either as daily dose or serum concentration) required to produce immunomodulation in humans are unknown. There is also lack of convincing evidence that such immunomodulation, if it were to occur, is likely to result in clinically relevant outcomes. There is however much speculation and theorising on this matter.

It is difficult to envisage how the available epidemiology information can be used quantitatively in risk assessment.

# Appendix A

## A1. Commonly used tests for assessing immune function

The immunomodulation tests recommended for use in animal studies are described by Luster *et al*. (1992, 1993), Tryphonas (2001), and ICH (2005).

Observational data such as measurement of circulating immunoglobulins or immunophenotyping of immune cells in blood or immune tissues are not considered to be robust endpoints for assessing immunomodulation. Assessment of the functionality of the immune system can be made by measuring antibody (Ig) response to antigen challenge, delayed hypersensitivity responses, activity of NK cells against a foreign cell or antibody production after inoculation with an infectious agent.

To assist the reader a brief description of some of the commonly employed procedures/tests for immune status determination after PFAS exposure of animals are described below.

### Splenocyte preparation

Spleens are aseptically processed into single cell suspensions, after gentle teasing apart with tweezers or scissors, with the use of sterile, frosted microscope slides. Cells may also be separated by gentle grinding in a hand homogeniser. Red blood cells are removed by adding red blood cell lysis buffer (ammonium chloride in Tris buffer) and the single cell splenocyte suspension washed several times. Cell viability, which needs to be confirmed before testing, is determined by trypan blue exclusion (measures cell membrane integrity) or the MTT reduction method (measures mitochondrial activity, MTT is a dye that changes colour after being metabolised in mitochondria).

### *Ex-vivo* lymphoproliferation

The lymphoproliferative assay involves treating animals, sacrifice and preparation of single cell splenocyte suspensions. Cells are cultured for varying times (eg. up to several days) in the presence of specific antigens or stimulants. Cell proliferation is measured using the MTT reduction test. The increase in optical density at 570nm is an indication of cell number increase after specific stimulation of the splenocyte suspension. Cell phenotyping is described below. Lymphoproliferation in response to mitogen stimulation is considered an observational endpoint because it is less predictive for immunotoxicity than functional immune measures (Luster *et al*. 1992).

Commonly used stimulants are:

- Concanavalin A (Con A), a plant lectin and T-cell activator.
- Phorbol myristate acetate, PMA, a specific activator of protein kinase C (PKC) and hence also T-cell activation, proliferation, and cytokine production (Fair *et al*. 2011).
- Lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria is a B-cell activator. Measure of T-dependent IgM responses.
- Trinitrophenol (TNP) -LPS conjugate, a T-cell independent activator. A measure of T-independent responses.

Less commonly used stimulants are:

- Anti-CD3 (T-cells).
- Anti-CD40 (B-cells).
- Keyhole limpet hemocyanin (KLH), T-cell dependent activator.

### Lymphocyte phenotyping

Immuno-phenotyping of peripheral blood lymphocytes or splenocytes uses specific monoclonal antibodies directed to cell-surface markers (different glycoproteins which are explicit for a particular type of lymphocyte). Flow cytometric techniques are used to separate and count the cells tagged with the antibody; often the mono-clonal antibodies have a fluorescent marker to facilitate quantitation. The specific glycoproteins are referred to as clusters of differentiation (CD) and are encoded by specific genes. Commonly $CD4^+$ (comprised mainly helper cells to the T cells; involved in the determination of the type of antigen response e.g cytotoxic or antibody based) and $CD8^+$ (comprised mainly

cytotoxic T lymphocytes) cells are measured (the number referring to the explicit glycoprotein). However there are many different CD cell surface glycoproteins each specifying a particular cell, some of these are measured in addition to the CD4 and CD8 cells.

#### *1.1.1.1 Note:*

There is significant variability in T-cell subsets in early childhood as the immune system undergoes expansion and maturation. Conversely, T-cell subsets in healthy adults whose immune systems have reached maturity are relatively stable. In adults the mean week-to-week variation in lymphocyte subpopulations is less than 5% (Tryphonas 2001).

**Antibody measurement**

For measurement of *in vivo* T-cell dependent antibody response (TDAR) mice are injected intravenously or intraperitoneally with antigen (horse or sheep red blood cells [SRBC]) and 4 – 6 days after immunisation blood removed and/or splenocyte suspensions made. Specific IgM and IgG1 antibodies produced against the antigen in serum or spleen cell suspension may be measured, usually by an Enzyme-Linked Immunosorbent Assay (ELISA) procedure. Antibody production is also measured *ex-vivo* in supernatant of cultured isolated splenocytes with and without stimulation by various antigens. Antigens not requiring T-cell involvement (eg. bacterial polysaccharides and lipopolysaccharides) are also used to measure T-cell independent antibody response (TIAR), ie. the role of B cells in generating antibodies.

IgM is important for the early or primary response after a single antigen challenge and IgG is a later response that is important in recognising the antigen following re-exposure. Antigen-specific IgM to a T-cell-dependent antigen (eg. SRBC) requires cooperation between T-cells, B-cells, and antigen-presenting cells (Luster *et al*. 1992). In humans, this antibody response can be examined by measuring antigen-specific antibody levels after vaccination. Measurement of total immunoglobulin levels (rather than antigen-specific IgM or IgG) is considered observational data (NTP 2016).

Assessing IgM TDAR and TIAR to an antigen are sensitive and predictive assays of immune function as it requires T cells, B cells, and antigen presenting cells to function properly in concert to elicit an antibody response (Luster *et al*. 1992). IL-4, IL-5, and IL-6 production by T cells is critical for a TDAR response. [Interleukins (IL) are cytokines mainly responsible for stimulating the immune response – see below for more details].

**Cytokine production**

Cytokines produced/released *ex-vivo* by splenocytes from treated hosts are usually measured using commercial kits made for the cytokine of interest (eg. Elispot or Elisa). Poly-clonal antibodies (suitably tagged to assist quantification) for the cytokine are incubated with serum or splenocytes.

Some cytokines are:

IL-γ: Signature cytokine from Th-1 (T helper) cells.

IL-2: From Th-1 cells. T-cell growth factor acts to stimulate growth and differentiation of T-cells, B-cells, and NK cells.

IL-4: Signature cytokine from Th-2 cells.

IL-10: From Th-2 cells. A negative immune regulator and inhibits the production of pro-inflammatory cytokines and mediators from macrophages. Has an important role in immune-regulation.

IL-6: IL-6 is produced for varied purposes by many cell types including muscle, macrophages, B-cells, and T-cells. It is a marker of inflammation, a necessary component for antibody production, and has roles in the hypothalamic–pituitary– adrenal axis (Fair *et al*. 2011).

**Plaque Forming Cell (PFC) assay**

Five days prior to the end of treatment animals are inoculated (intraperitoneally or intravenously) with SRBC. Splenocytes should mount IgM response to these foreign cells. At sacrifice splenocyte suspensions are prepared and incubated in culture dishes in the presence of complement (a mixture of serum proteins which assists in the immune response) and with SRBC. The extent of SRBC specific IgM antibodies produced *in vivo* after inoculation with SRBC is measured as the number of plaques (clear areas on a background of red) since the antibodies will initiate lysis of SRBC in the co-incubation.

Decreases in the plaque-forming cell (PFC) response are considered predictive of decreased host resistance (Luster *et al*. 1992, 1993; Tryphonas 2001, Selgrade 1999), which in turn can lead to increased susceptibility to infection.

**Systemic delayed-type hypersensitivity (DTH) response**

The test involves treating animals (mice usually) with the compound of interest. Towards the end of the treatment period the animals are primed with intravenous or intraperitoneal antigen (eg. SRBC) as well as maintaining compound treatment. At the end of treatment an intradermal injection of antigen(s) is given (usually to a footpad) and erythema and/or oedema at the injection site determined 24–48 hr later. The inflammatory reaction is a lymphocyte- and macrophage-dependent delayed-type hypersensitivity response (Tryphonas 2001). A variety of antigens other than SRBC may be used.

In addition, at the end of the antigen priming period (ie. prior to antigen challenge) blood may be taken from a parallel treated group of animals for analysis of antigen-specific IgM (eg. SRBC-specific IgM) as well as from the antigen challenged group of animals.

**NK cell activity**

Plasma, or washed single cell splenocyte suspensions are incubated with a foreign target cell and NK cell function determined by the number, or proportion, of the target cells killed. A variety of target cells have been used, eg. K562 cells, Yac-1 cells. Quantitating target cell death is achieved in a number of ways. For example preloading with $^{51}$chromium (Cr) and measuring the release of radiolabel in culture supernatant after incubation with NK cells (Tryphonas 2001, Peden-Adams *et al*. 2008), or release of cytoplasmic enzyme (eg. LDH), measured as utilisation of its substrate lactic acid, from the target cell (Zheng *et al*. 2009).

NK cells produce numerous cytokines such as tumour necrosis factors α and β, interferons α and β, granulocyte-macrophage colony-stimulating factor, and interleukin-3 upon immune stimulation, all of which have a profound effect on immune reactivity.

## A2. Epidemiology endpoints

The majority of end points assessed in humans, including determination of the total serum Ig classes and subclasses, quantification of peripheral blood leukocytes and T-lymphocyte subsets, the lymphoproliferative activity of peripheral blood leukocytes in response to mitogens, NK cell activity, and monocyte function are easily investigated in *in vitro* systems using peripheral blood from humans known to be exposed to environmental contaminants (Luster *et al*. 1992, Tryphonas 2001).

Generally epidemiological data are mostly restricted to:

- Observational data, such as circulating immunoglobulin levels, lymphocyte counts, and cytokine levels. However circulating antibodies fluctuate over relatively short time periods and vary with age of the individual.
- Incidence estimates of disease associated with possible suppression of the innate immune pathways.
- Altered responses of the adaptive immune system:
  - » Clinical manifestation of hypersensitivity (allergy, asthma, eczema etc).
  - » Suppression of specific antibody response to vaccination. A variety of vaccinations have been assessed in studies of the immunomodulation effects of PFASs.
  - » Incidence of common infectious diseases (eg. common cold, flu, Otitis media, gastroenteritis).

Statistical analysis is often not with a 'control' population but between measurement strata of the study population whereby associations are found when the lowest PFAS exposure (eg. the lowest quartile) is compared with the highest PFAS exposure (eg. highest quartile). But the different strata are usually over a very low and narrow serum concentration range, 0.002 – 0.05 mg/L for PFOS, which is within background serum concentrations. No doubt this contributes to the weak associations and inconsistency between studies. As does different assessment methodologies for immunomodulation. Furthermore, the statistical analysis in many studies is not easily understood with respect to how a particular PFAS was dissected out for a positive association but other co-exposure PFASs show no association. It is also noted that many of the associations are weak, the effects small and of questionable clinical significance.

Furthermore there are many chemicals known to have immunomodulating effects[27], and for some of which there may be co-exposure with PFASs but have not been addressed in epidemiology studies for PFASs.

27 Chemical classes known to have immunomodulating effects include polycyclic aromatic hydrocarbons, halogenated aromatic hydrocarbons, organochlorine and organophosphorous pesticides, and heavy metals (Vial *et al*. 1996).

# Appendix B: Summary of immunomodulation animal studies

Abbreviations and symbols used in this Appendix are:

## Symbols

| | |
|---|---|
| ↓ | Decrease relative to control |
| ↓↓ | Marked decrease relative to control |
| ↑ | Increase relative to control |
| Sl↑ | Slight increase relative to control |
| ↔ | No change relative to controls |
| ♂ | Male |
| ♀ | Female |

## Units

| | |
|---|---|
| **kg** | Kilogram |
| **L** | Litre |
| **mL** | Millilitre |
| **µg** | Microgram |
| **ng** | Nanogram |

## Abbreviations

| | |
|---|---|
| **APFO** | Ammonium perfluorooctanoate, PFOA is the anion to this substance |
| **bw** | Body weight |
| **B** | B-cell |
| **Conc** | Concentration |
| **d** | Day |
| **DST** | Delayed hypersensitivity test |
| **GD** | Gestation day |
| **Ig** | Immunoglobulin |
| **IL** | Interleukin |
| **lPFOS** | Linear perfluorooctane sulphonate. |
| **lAPFO** | Linear ammonium perfluorooctanoate. |
| **KO** | Knock out |
| **ND** | Not determined |
| **NK** | Natural killer cell |
| **PFC** | Plaque forming assay |
| **PND** | Postnatal day |
| **Prolif** | Proliferation |
| **SRBC** | Sheep red blood cell |
| **T** | T-cell |
| **TAD** | Total administered dose |
| **TDAR** | T-cell dependent antibody response |
| **TIAR** | T-cell independent antibody response |
| **wt** | Weight |
| **WT** | Wild type |

## Table B1: Animal immunomodulation studies with PFOS

(An empty cell in the table indicates no data for that endpoint; TAD = Total Administered Dose; Ab = Antibody; ↓ Decrease, ↑ Increase, ↔ No change cf control).

| Study | Dose | | Serum conc (mg/L) | Effect | | | Cellularity | | Ab | Cytokine | Cell activity | PFC assay (Igm) | Other / comment |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Body Wt | Spleen Wt | Thymus Wt | # | Type (Spleen) | | | | | |
| **Dong *et al.* 2009**. Mouse C57BL/6♂ **Gavage 60d** IPFOS K+ in water 0.02% Tween 80 | µg/kg/d | TAD mg/kg | | | | | | | | | | | |
| | 0 | 0 | 0.048 ± 0.01 | | | | | | | | | | |
| | 8.33 | 0.5 | 0.674 ± 0.17 | ↔ | ↔ | ↔ | | | | | ↔ | ↔ | ↔ |
| | 83.3 | 5 | 7.13 ± 1.0 | ↔ | ↔ | ↔ | ↔ | ↔ | | | ↑ NK | ↔ | ↑ liver wt |
| | 416.7 | 25 | 21.64 ± 4.4 | ↓ | ↓ | ↓ | ↓ | ↓CD4 | | | ↔ NK | ↓ | ↑ liver wt |
| | 833.3 | 50 | 65.43 ± 11.7 | ↓ | ↓ | ↓ | ↓ | ↓CD4/CD8 ↓B | | | ↓NK<br>↓ B Prolif | ↓ | ↑ liver wt |
| | 2083 | 125 | 120.67 ± 21.8 | ↓ | ↓ | ↓ | ↓ | ↓CD4/CD8<br>↓B | | | ↓NK<br>↓ B Prolif<br>↓T Prolif | ↓ | ↑ liver wt |

Dong *et al*. (2009) shows decreased spleen and thymus weight at PFOS doses ≥417 µg/kg/d for 60d (total dose 25 mg/kg) and corresponding serum concentration of ≥21.6 mg/L. At these serum concentrations there are also decreased body weight (loss and gain) and increased liver weight (no histology but presumed PPARα mediated hypertrophy).

Changes in immune organ cellularity occurred in association with toxicity in these organs (decreased organ weight).

Increased NK activity, decreased functionality with plaque forming assay and increased liver weight all occurred at the second to lowest dose (7.1 mg/L PFOS, serum).

NK activity was increased at 7.1 mg/L, unchanged at 21.6 mg/L, and decreased at 65.4 mg/L and above, ie. showed an inverted U-shape dose response.

Based on the decreased PFC the immune-NOEL = 0.67 mg/L and LOEL = 7.1 mg/L. There was a good dose-response. ↑ liver wt is likely adaptive & PPARα mediated. Although not to GLP, the study was well conducted. According to Dong *et al*. (2009) male mice were chosen to avoid hormonal changes associated with ovulation and menstruation, which can influence the immune system. Laboratory mice do not menstruate.

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dong *et al.* 2011** Mouse C57BL/6♂ **Gavage 60d** IPFOS K+ 0.02% Tween 80 | 0 | 0 | 0.05 ± 0.01 | | | | | | | | | | |
| | 8.3 | 0.5 | 1.07 ± 0.11 | ↔ | ↔ | ↔ | | ↔ | ↔ | ↔ | | | ↔ |
| | 16.7 | 1 | 2.36 ± 0.47 | ↔ | ↔ | ↔ | | ↔ | ↔ | ↔ | | ↔ DST | ↔ |
| | 83.3 | 5 | 10.75 ± 0.82 | ↔ | ↔ | ↔ | | ↔ | ↓ IgM | ↑ IL-4 | Ig serum assays are SRBC specific. | ↔ DST | ↔ |
| | 416.7 | 25 | 22.64 ± 2.29 | ↔ | ↔ | ↔ | | ↔ | ↓ IgM | ↑ IL-4 | | ↔ DST | ↑ liver wt |
| | 833.3 | 50 | 51.71 ± 3.81 | ↓ | ↓ | ↓ | | ↓ IL-2⁺<br>↑ IL-10⁺ | ↓ IgM<br>↑ IgG<br>↑ IgE⁺ | ↑ IL-4<br>↓ IFN-γ | IL assays in *ex vivo* splenocytes | ↔ DST<br>DST; *In vivo* challenge & measure foot pad | ↑ liver wt |

Dong *et al*. (2011) has similar experimental design as Dong *et al*. (2009) but animals given antigenic stimulus (1x i.v. SRBC 7d before sacrifice) and circulating SRBC–IgM and delayed hypersensitivity test (DST, SRBC injection into foot pad) instead of PFC. Immune cell activity tests conducted in Dong *et al*. (2009) were replaced by interleukin release assays for immune system function assessment. Serum PFOS concentrations are similar in both studies. Decreased circulating SRBC specific-IgM (immediate immune response by B cells to SRBC) and increased IgG (secondary memory response), only at top dose, was observed. At top dose there was also increased SRBC specific IgE but no delayed sensitivity response. Increased IL-4 and decreased IFN-γ from isolated splenocytes suggests excess Th2 (effector) and deficient Th1 responses. Perhaps making the animals more susceptible to infections. Based on decreased IgM and increased IL-4 immune-NOEL = 2.36 & LOEL = 10.8 mg/L.

| Study | Dose | | Serum conc (mg/L) | Effect | | | Cellularity | | Ab | Cytokine | Cell activity | PFC assay (Igm) | Other / comment | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Body Wt | Spleen Wt | Thymus Wt | # | Type (Spleen) | | | | | | |
| **Dong *et al.* (2012a)** Mouse C57BL/6♂ **Gavage 60d** IPFOS K+ in water 0.02% Tween 80 | µg/kg/d | TAD mg/kg | | | | | | | | | | | | |
| | 0 | 0 | 0.04 ± 0.01 | | | | | | | | | | | |
| | 16.7 | 1 | 4.35 ± 0.63 | ↔ | ↔ | ↔ | ↔ | | | | | | ↔ | |
| | 83.3 | 5 | 8.21 ± 1.15 | ↔ | ↔ | ↔ | ↑ Apopt's | | | | | | ↑ liver wt<br>↓ MMP Spleen | |
| | 833.3 | 50 | 59.74 ± 12.2 | ↓ | ↓ | ↓ | ↓ #<br>↑ Apopt's | | | | | | ↑ liver wt<br>↓ MMP Spleen | |
| In Dong *et al*. (2012a) a variety of end points have been measured (cell viability, cell cycle, cell apoptosis/necrosis, mitochondrial membrane potential [MMP], gene expression eg. p53]). Increased apoptosis (decreased cell viability) in spleen and thymus cell suspensions at ≥ 5 mg/kg TAD and MMP splenocytes decreased. Immuno-NOEL (↑ splenocyte apoptosis, ↓ MMP) = 4.35 mg/L. (↑ liver wt is likely adaptive & PPARα mediated).<br>NOEL = 4.35 mg/L based on increased apoptosis and decreased MMP in splenocytes. LOEL = 8.21 mg/L. | | | | | | | | | | | | | | |
| **Dong *et al.* (2012b)** Mouse C57BL/6♂ **Gavage 60d** IPFOS K+ in water 0.02% Tween 80 | 0 | 0 | 0.04 ± 0.01 | | | | | | | | | | | |
| | 8.3 | 0.5 | 0.58 ± 0.19 | ↔ | ↔ | ↔ | | | | ↔ | ↔ | | ↔ | |
| | 16.7 | 1 | 4.35 ± 0.63 | ↔ | ↔ | ↔ | | | | ↔ | ↔ | | ↔ | |
| | 83.3 | 5 | 8.21 ± 1.15 | ↔ | ↔ | ↔ | | | | | ↑ IL-1β<br>↔ Others | Macrophages not splenocytes | ↑ liver wt | |
| | 416.7 | 25 | 24.53 ± 5.56 | ↓ | ↓ | ↓ | | | | | ↔ IL-β | | ↑ liver wt | |
| | 833.3 | 50 | 59.74 ± 12.2 | ↓ | ↓ | ↓ | | | | | ↑ IL-1β<br>↑ IL-6 | Cytokines ↑ *ex-vivo* with *in vivo* exposure with & without iv LPS | ↑ liver wt | |
| | 2,083 | 125 | 114.19 ± 23.72 | ↓ | ↓ | ↓ | | | | | ↑ IL-1β<br>↑ IL-6 | | ↑ liver wt | |
| After treatment with PFOS Dong *et al*. (2012b) investigated inflammatory response of isolated splenocytes and peritoneal macrophages co-incubated with LPS after oral PFOS treatment by measuring release of proinflammatory cytokines (TNF-α, IL-1β, IL-6) from the cells, or their presence in serum after *in vivo* intravenous LPS.<br>At 8.21 mg/L, there was increased IL-1β release from unstimulated isolated peritoneal macrophages and in macrophages after intravenous LPS, but not from splenocytes. However gene expression for IL-1β was increased in splenocytes.<br>At ≥ 24.5 mg/L there was increased release of all the proinflammatory cytokines from peritoneal macrophages, from splenocytes ≥ 59.7 mg/L, with or without LPS stimulation.<br>However serum cytokine increases were only observed at ≥ 59.7 mg/L with or without LPS intravenous injection.<br>Notwithstanding the *ex-vivo* increase of a single cytokine (IL –1β) at 8.21 mg/L from peritoneal macrophages (? Chance finding), the overall NOEL for pro-inflammatory cytokine effects = 24.5 mg/L, but for effects on body and immune organ weights = 8.2 mg/L. LOAEL = 24.5 mg/L based on body & immune organ weight changes. (↑ liver wt at 8.21 mg/L is likely adaptive & PPARα mediated).<br>Some techniques same as Qazi *et al*. (2010).<br>The same serum concentrations for same doses as in Dong *et al*. (2012a) suggest this is the same study but reporting different endpoints. | | | | | | | | | | | | | | |
| **Zheng *et al.* (2009).** Mouse C57BL/6♂ **Gavage 7d** IPFOS K+ in water 0.02% Tween 80 | µg/kg/d | TADmg/kg | | | | | | | | | | | | |
| | 0 | | <LOR | | | | | | | | | | | |
| | 5,000 | 35 | 110 ± 6.18 | ↔ | ↔ | ↔ | ↔ | ↔ | | | ↔ NK, ↔ B cell ↓ T cell prolif'n | ↓ | Liver wt | ↑ 34% |
| | 20,000 | 140 | 281 ± 16.3 | ↓ | ↓ | ↓ | ↓ S,T | ↓ CD4<br>↓ CD8<br>↓ B | | | ↓NK, T & B cell | ↓ | | ↑ 79% |
| | 40,000 | 280 | 338 ± 23.9 | ↓↓ | ↓ | ↓ | ↓ S,T | | | | ↓NK, T & B cell | ↓ | | ↑ 117% |

| Study | Dose | | Serum conc (mg/L) | Effect | | | Cellularity | | Ab | Cytokine | Cell activity | PFC assay (Igm) | Other / comment | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Body Wt | Spleen Wt | Thymus Wt | # | Type (Spleen) | | | | | | |
| Zheng *et al*. (2009) uses PFOS doses that achieve very high serum concentrations. At the highest doses there is significant decreased food intake and decreased body weight (ie. bw loss). All doses decreased plaque formation, an indication of reduced IgM production by B-cells. | | | | | | | | | | | | | | |
| Based on decreased PFC & ex vivo T cell proliferation the LOEL is 5 mg/kg/d, since this is the lowest dose no NOEL was identified. | | | | | | | | | | | | | | |
| **Zheng *et al.* (2011).** Mouse C57BL/6♂ **Gavage 7d** IPFOS K+ in water 0.02% Tween 80 | µg/kg/d | TADmg/kg | | | | | | | | | | | | |
| | 0 | | <LOR | | | | | | | | | | | |
| | 5,000 | 35 | 97.3±7.6 | | ↔ | ↔ | ↔ | | ↓ serum IgM<br>↑ IgG | ↑ IL-4 | | | Liver wt | ↑ 34% |
| | 20,000 | 280 | 250±20.1 | 280<br>↓ serum IgM<br>↔ IgG | ↓↓ | ↓ | ↓ | | ↓ serum IgM<br>↔ IgG | ↑ IL-4<br>↓ IL-2<br>↔ IL-10<br>↓ IFN-γ | | | | ↑ 79% |
| Zheng *et al*. (2011) has same experimental design as Zheng *et al*. (2009) and other papers in research group (Dong *et al*. 2009, 2011) but reports different assays. | | | | | | | | | | | | | | |
| *Ex-vivo* decreased release IL-2 (T-cell growth factor), decreased IFN-γ (Th-1 cells) from splenocytes (no *in vitro* stimulation) and increase IL-4 (Th-2) cells suggested to the authors a shift in T-cell balance away from Th-1 co-stimulator signals for NK cells (decreased at same dose in Zheng *et al*. 2009) and perhaps animals were more vulnerable to infections, as reflected in decreased circulating IgM at the high dose. But note there was increased IgG at the lower dose. LOEL = 5 mg/kg/d (97 mg/L serum), since this is the lowest dose no NOEL was identified. | | | | | | | | | | | | | | |
| **Mollenhauer *et al.* (2011).** Mouse B6C3F1♀ **Gavage 28d** IPFOS in water plus 0.5%Tween 20 | µg/kg/d | TAD mg/kg | | | | | | | | | | | | |
| | 0 | 0 | | | | | | | | | | | | |
| | 33.1 | 1 | | ↔ | ↔ | | ↔ | | | ↑ serum IL-6<br>↓serum TNF-α | | | ↔ | |
| | 99.3 | 3 | | ↔ | ↔ | | ↔ | | | ↓ IL-6<br>↔ TNF-α | | | ↔ | |
| | 9,930 | 300 | | ↓ | ↓ | | ↔ | | | " | | | ↑ liver wt | |
| This study investigates inflammatory cytokines (IL-6 and TNF-α) in serum and release *ex-vivo* from splenocytes and peritoneal macrophages after oral PFOS, with and without *in vivo* challenge with intraperitoneal LPS (1 hr before sample collection), or *in vitro* incubation of cells with LPS. Serum PFOS not measured. | | | | | | | | | | | | | | |
| After *in vivo* LPS challenge: Serum TNF-α concentrations significantly decreased with 1 mg/kg but no change with 3 or 300 mg/kg TAD. There was an ↑ in serum IL-6 at 1 mg/kg and ↓ at 3 mg/kg mirroring the numbers of cells expressing intracellular IL-6. Production of IL-6 by *ex-vivo* macrophages (no *in vitro* challenge) ↑ only at 300 mg/kg TAD, but TNF-α was ↔. In contrast production of TNF-α by peritoneal macrophages from unchallenged mice (with *in vitro* challenge) ↑ only at 300 mg/kg TAD, IL-6 was ↔. | | | | | | | | | | | | | | |
| The authors concluded serum inflammatory cytokine production by macrophages is not altered at environmentally relevant PFOS exposures, but may be at very high doses. | | | | | | | | | | | | | | |
| LOEL = 1 mg/kg based on ↑ circulating IL-6 after LPS challenge. But usefulness limited due to lack of response at higher doses, opposite effects on IL-6 and TNF- α from macrophages depending on whether challenge was *in vivo* or *in vitro*, and no internal dose measurement. | | | | | | | | | | | | | | |

| Study | Dose | | Serum conc (mg/L) | | Effect | | | Cellularity | | Ab | Cytokine | Cell activity | PFC assay (Igm) | Other / comment |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Body Wt | Spleen Wt | Thymus Wt | # | Type (Spleen) | | | | | |
| **Peden-Adams *et al.* (2008).** Mouse B6C3F1♂ & ♀. **Gavage 28d** IPFOS K+ in water with 0.5% Tween 80 | µg/kg/d | TAD mg/kg | Serum conc | | | | | | | | " | | | |
| | | | ♂ | ♀ | | | | | | | | | | |
| | 0 | 0 | 0.012 | 0.016 | | | | | | | | | | |
| | .166 | 0.005 | 0.018 | ND | ↔ | ↔ | ↔ | ↔ | ↔ | | | | ↔ | ↑ liver wt |
| | 1.66 | 0.05 | 0.09 | 0.09 | ↔ | ↔ | ↔ | ↔ | ↔ | | | | ↓♂ ↔♀ | " |
| | 3.31 | 0.1 | 0.13 | 0.12 | ↔ | ↔ | ↔ | ↔ | spleen CD4/ CD8 differences in ♂ & ♀ | | | | | " |
| | 16.6 | 0.5 | ND | 0.67 | ↔ | ↔ | ↔ | ↔ | " | | | ↑ NK ♂ ↔ NK ♀ | ↓♂ ↓♀ | " |
| | 33.1 | 1 | ND | ND | ↔ | ↔ | ↔ | ↔ | " | | | | | " |
| | 166 | 5 | > calibration | | ↔ | ↔ | ↔ | ↔ | ↔ T cell ↔ B cell | | | | | " |
| Noted serum concentrations not different between male & female, good interlab agreement with a standard. Splenocyte proliferation to Con A- (T-cell) or LPS-stimulation (B-cell) not altered. NK activity not altered in females but increased in males ≥ 0.5 mg/kg TAD (0.67 mg/L). The number of plaque forming cells (PFC) (implying decreased SRBC-specific IgM production but specific SRBC-IgM was not measured in plaque assay nor *in vivo*) was lower in males at ≥0.09 mg/L and female ≥0.67 mg/L. Because serum TNP specific IgM was suppressed after inoculating mice with TNP-LPS (a T-cell independent antigen) the authors concluded the decrease in PFC and SRBC-IgM was an effect on B-cells. NK-cell activity was not affected in females but it was significantly increased in males at ≥ 0.67 mg/L. No change in organ weights is attributed to lower serum levels, this is same as other studies. The NOEL (based on ↓ plaque forming cells) = 0.018 mg/L males & 0.12 mg/L females and LOEL = 0.09 mg/L males and 0.67 mg/L females. | | | | | | | | | | | | | | | |
| The authors indicate the tests used are consistent with NTP tiered immunotoxicity scheme (Luster *et al*. 1988, 1992) and US EPA Harmonised Test Guidelines. | | | | | | | | | | | | | | |
| They speculate PPARα may be mediating the effects, and gender differences due to impact on sex hormones. This study has the lowest serum concs linked to a functional effect. | | | | | | | | | | | | | | |
| **Guruge *et al.* (2009)** Mouse B6C3F1♀ **Gavage 21d** IPFOS in water plus 0.02% Tween 80 | µg/kg/d | TAD mg/kg | | | | | | | | | | | | |
| | 0 | 0 | 0.002 ± 0.0003 | | | | | | | | | | | |
| | 5 | 0.1 | 0.19 ± 0.014 | | ↔ | ↔ | ↔ | | | | | ↔ Survival ↓ Body wt post influenza A infection | | ↔ liver wt |
| | 25 | 0.53 | 0.67 ± 0.047 | | ↔ | ↔ | ↔ | | | | | ↓ Survival ↓ Body wt | | ↔ liver wt |
| It is noted plasma PFOS concentrations are much lower than would be expected from comparison to Dong group. No difference in clearance in male & female mice, t½ ~37d (Chang *et al*. 2012) and Peden-Adams *et al*. (2008) showed no difference when both genders given same gavage dose for 28d. Also noted there is no adaptive ↑ liver wt at these doses that has been observed in other studies. After PFOS treatment mice were intranasally infected with influenza A while under anaesthesia. There was a significant dose trend for increased mortality with top dose different to controls, but no difference in time to death. No NOAEL based on increased influenza A effects in treated mice. LOAEL = 0.19 mg/L. Note the different plasma levels (same strain/gender as Fair *et al*. 2011 and Mollenhauer *et al*. 2001) and lack of spleen function assessment make it difficult to compare with other studies, need confirmation for increased infectivity at the reported plasma levels. | | | | | | | | | | | | | | |

| Study | Dose | | Serum conc (mg/L) | Effect | | | Cellularity | | Ab | Cytokine | Cell activity | PFC assay (Igm) | Other / comment |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Body Wt | Spleen Wt | Thymus Wt | # | Type (Spleen) | | | | | |
| **Fair *et al.* (2011).** Mouse B6C3F1♀ **Gavage 28d** lPFOS in water plus 0.5% Tween 80 | µg/kg/d | TADmg/kg | | | | | | | | | | | |
| | 0 | 0 | <LOQ | | | | | | | | | | |
| | 3.31 | 0.1 | <LOQ | ↔ | ↔ | ↔ | ↔ | ↔ T & B-cell surface markers | | ↑ IL-6 from B-cells, ↔ IL-4, IL-5, IL-6 from T-cells *ex-vivo* | | | ↔ Organ wts |
| | 16.6 | 0.5 | 1.16±0.09 | ↔ | ↔ | ↔ | ↔ | | | Not done | | | ↔ Organ wts |
| | 33.1 | 1 | 2.15±0.55 | ↔ | ↔ | ↔ | ↔ | | | | | | ↔ Organ wts |
| | 166 | 5 | 12.47±0.61 | ↔ | ↔ | ↔ | ↔ | | | Not done | | | ↓ Uterine wt sl ↓ cholesterol |
| Body and organ weight (except uterus), haematology, histopathology was unchanged at each dose. Except for non-significant increase in glucose (31%) and decrease in cholesterol (27%) at 12.47 mg/L, serum clinical chemistry (including thyroid hormones) was unchanged. Noted TSH and free thyroid hormone not measured. No overt toxicity in treated animals. Isolated splenocytes used for immune assessments including cell surface markers, cytokine production following specific stimulation. | | | | | | | | | | | | | |
| B-cell numbers, sub-types and numbers of cells expressing MHC-II and CD40 surface markers, which are found on antigen-presenting cells, were not altered. | | | | | | | | | | | | | |
| Decreases in absolute numbers of CD4+ cells seen in Peden-Adams *et al*. (2008) not found in this study. Authors consider previous finding likely a transient effect. | | | | | | | | | | | | | |
| Based on increased IL-6 release from *in vitro* stimulated *ex-vivo* B-cell splenocytes (exposed *in vivo*) a NOEL was not demonstrated, LOEL = 0.1 mg/kg TAD but PFOS serum concentrations were <LOQ (0.001 mg/L) which creates uncertainty regarding the veracity of the effect at these low doses. Previous TAD of 0.1 mg/kg has given serum concentrations of 0.19 mg/L (Guruge *et al*. 2009) and 0.12 mg/L (Penden-Adams *et al*. 2008) in female mice of this strain. | | | | | | | | | | | | | |
| **Qazi *et al.* (2009a).** Mouse C57BL/6♂ **Diet 10d** | %diet(w/w) | TADmg/kg | | | | | | | | | | | |
| | 0 | | 0.029±0.01 | | | | | | | | | | |
| | 0.001 | ~25 | 50.8±2.5 | ↔ | ↔ | ↔ | ↔ | ↔ | | | | | ↑ liver wt |
| | 0.005 | ~100 | 96.7±5.2 | ↔ | ↔ | ↔ | ↔ | ↔ | | | | | ↑ liver wt |
| | 0.02 | ~260 | 340±16 | ↓ ~25% | ↓↓ No structural change | ↓↓ ↓ Histologically cortex small, v. few cells. Cort/med junction not apparent | | ↓ in all cell subpop phenotype | | | | | ↑ liver wt ↓ Fat wt |
| | 0.05 | ~100 | NR | ↓↓ | NR | NR | NR | NR | | | | | Lethargy & poor grooming |
| | 0.1 | | NR | ↓↓ | NR | NR | NR | NR | | | | | " |
| Relationship between dose and serum concentration is not linear. Steep dose response for effects (↓body [↓ food intake] & immune organ wts, immune cellularity), liver hypertrophy most sensitive endpoint. PFOS effects less than PFOA at same dose (PFOA 2x less serum conc). Reduced food intake accounts for at least part of the immunomodulatory responses. In PPARα – null mice ↑ liver wt, no change in thymus or fat wts. Effects on splenocytes almost totally eliminated, changes in thymocytes numbers and cell subpopulations partially or almost totally attenuated. The authors conclude immunomodulation caused by PFOS is a high-dose phenomenon partially dependent on PPARα. Immuno NOAEL = 96.7 mg/L (decreased organ wt & splenic/thymic cellularity), LOAEL = 340 mg/L. Noted no immune functionality tests done. NR = Not Reported. | | | | | | | | | | | | | |

| Study | Dose | | Serum conc (mg/L) | | Effect | | | Cellularity | | Ab | Cytokine | Cell activity | PFC assay (Igm) | Other / comment |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Body Wt | Spleen Wt | Thymus Wt | # | Type (Spleen) | | | | | |
| **Qazi *et al.* (2010a).** Mouse C57BL/6♂ **Diet 10d** | %diet(w/w) | µg/kg/d | TADmg/kg | | | | | | | | | | | |
| | 0 | | | | | | | | | | | | | |
| | 0.005 | | ~100 | 125.8 ± 3.9 | ↔ | ↔ | ↔ | ↔ Spleen | | | ↓ TNF-α, IFN-γ, IL-4 in liver | ↔ in IgM & IFN-γ response to LPS or Con A | | ↑ liver wt (centrilobular hypertrophy) ↓ serum TGs & cholesterol ↑ serum ALP |
| This study investigated the effect of PFOS & PFOA on hepatic immune status. Numbers of hepatic cells that appear immunophenotypically to be erythrocyte progenitors were increased, other intrahepatic immune cells were unaffected. There were decreased cytokines in the liver, but ex vivo production of IgM by hepatic B cells and IFN-γ by hepatic T cells with specific stimulators (LPS and Con A, respectively) was not statistically different from controls, but appeared to be larger in PFOS treated animals. Data for splenocytes not included. | | | | | | | | | | | | | | |
| Study not suitable for NOEL determination. | | | | | | | | | | | | | | |
| **Qazi *et al.* (2010b).** Mouse B6C3F1♂ **Diet 28d** | %diet(w/w) | µg/kgbw/d | TADmg/kg | | | | | | | | | | | |
| | 0 | | 0 | 0.04 ± 0.002 | | | | | | | | | | |
| | 0.000156 | 250 | 5.55 | 11.6 ± 0.2 | ↓ bw gain but ↔ Food intake | ↔ | ↔ | ↔ | ↔ (also for thymus) | ↔ serum IgM & IgG specific for SRBC. ↔ serum IgM specific for TNP-LPS | | | ↔ with SRBC | ↑ liver wt |
| TAD ~ same as maximum in Peden-Adams *et al*. (2008) (ie. 5 mg/kg, LOEL) over the same exposure time (28d) and above NOEL (1 mg/kg TAD) of Dong *et al*. (2011, 2012a,b) with serum concentrations of approximately 2 – 4.5 mg/L. | | | | | | | | | | | | | | |
| On d23 animals injected with T-cell dependent (SRBC, intraperitoneally) or independent (TNP-LPS) antigen. At end of 28d exposure circulating IgM and IgG specific for injected antigens measured. Also splenic & thymic cell phenotypic sub-populations (eg. CD4–/CD8–,CD4+/CD8+, Th, Tc, Tγδ, NKT, NK, B-cells, macrophages) in non-immunised mice. | | | | | | | | | | | | | | |
| No differences from controls for any of the immune parameters. The humoral response in PFOS treated animals to antigens was appropriate and same as controls. | | | | | | | | | | | | | | |
| Authors concluded, in contrast to gavage studies, dietary exposure to environmentally relevant doses does not compromise humoral immune responses. | | | | | | | | | | | | | | |
| Immuno NOEL = 11.6 mg/L. As this was the only dose used the NOEL could be higher. | | | | | | | | | | | | | | |
| Humoral response measured 4 ways; proportion of plasma cells in spleen, number secreting SRBC-IgM, plaque assay, and circulating SRBC-IgM & IgG. | | | | | | | | | | | | | | |

| Study | Dose | | Serum conc (mg/L) | | Effect | | | Cellularity | | Ab | Cytokine | Cell activity | PFC assay (Igm) | Other / comment |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Body Wt | Spleen Wt | Thymus Wt | # | Type (Spleen) | | | | | |
| **Lefebvre *et al.* (2008).** Rat Sprague-Dawley ♂ & ♀ **Diet 28d** | mg/kg diet | | | | | | | | | | | | | |
| | | ♀ | ♂ | ♀ | | | | | | | | | | |
| | 0 | 0 | 0.47 | 0.95 | | | | | | | | | | ↔ |
| | 2 | 0.15 | 0.95 | 1.5 | ↔ | ↔ | ↔ | | ↔ | | | ↔ *ex-vivo* splenocyte proliferation with Con A or LPS. ↔ Delayed hyper-sensitivity response. | | |
| | 20 | 1.43 | 13.5 | 15.4 | ↔ | ↔ | ↔ | | ↔ | | | | | |
| | 50 | 3.73 | 20.9 | 31.9 | ↓ gain ♂♀ | ↔ | ↔ | | ♂ Lymphocyte apoptosis in thymus. | | | | | |
| | 100 | 7.58 | 29.9 | 43.2 | " | ↓ ♂ Spleen to body wt ↔ ♂, ↑ ♀ | Thymus to body wt ↔ ♂♀ | | | | | | | |

Based on the authors daily dose (mg/kg/d) calculated TAD over the exposure period is approximately 5, 40, 100 and 200 mg/kg bw for 2, 20, 50 and 100 mg PFOS/kg diet. Study has two components: 1. General toxicology, serum lymphocyte phenotyping & Ig analysis. 2. Immune challenge & serum Ig analysis.

1. At 100 mg/kg diet, males had decreased spleen wt accompanied by increased lymphocyte apoptosis, but only occasionally in females. No histological changes. Increased apoptosis in male thymus at 50 & 100 mg/kg, and females at 100 mg/kg diet. No difference from control in circulating leukocytes, lymphocytes or subclasses. But trend for increased T & Th cells and decreased B cells for both male & females. Decreased circulating IgG1 at 2 & 20 mg/kg, but not at higher doses for males (trend for other IgG's to increase), increased IgM & IgG2c in females at 100 mg/kg diet.

2. No difference from controls for *ex-vivo* stimulation of splenocytes with Con A or LPS (T-cell & B- cell stimulation). Rats immunised with T-dependent keyhole limpet hemocyanin (KLH) (a T-independent lymphocyte activator) and subsequently challenged with KLH. There was a trend in males for increased KLH-specific IgG, no changes in females from treated and challenged controls. No changes from control animals for delayed hypersensitivity responses to KLH (footpad swelling). The *ex-vivo* splenocyte stimulation and *in vivo* response to immunisation indicate the treated animals were functionally immunocompetent.

If decreased circulating IgG1 in males is considered to be a real effect the immune-LOEL is 1 mg/L, this coincides with increased liver to body weight ratios in males and there is no NOEL for the study. However

- There was not a clear dose response (change not evident at exposures ≥ 20 mg/L) but perhaps this may be a non-monotonic dose response.
- In male rats the trend was for other Ig's to increase, but at each exposure there was no significance.
- The effect is not observed in female rats.
- The decrease is not associated with functional immune response with *in vivo* challenge with antigen. The animals were equally immunocompetent as control animals.
- *Ex-vivo* splenocyte responses to antigen stimulation were not altered.

In considering the above it is suggested lymphocyte apoptosis in thymus of males (LOEL 20.9 mg/L) may be a more appropriate endpoint for a NOEL in this study, ie. NOEL of 13.5 mg/L.
In females the NOEL was 43.2 mg/L.

The authors suggest immune responses in the rat may, in part, be secondary to hepatic changes.

| Study | Dose | | Serum conc (mg/L) | Effect | | | Cellularity | | Ab | Cytokine | Cell activity | PFC assay (Igm) | Other / comment |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Body Wt | Spleen Wt | Thymus Wt | # | Type (Spleen) | | | | | |
| **Keil *et al.* 2008** Mouse C57BL/6N♀ **Gavage Develop'lGD 1-17** lPFOS in water plus 0.5% Tween 80 | mg/kg/d | TAD mg/kg calculated | Extra-polated | | | | | | | | | | |
| | 0 | | | | | | | | | | | | |
| | 0.1 | 1.7 | ~ 1 | ↔ | ↔ | ↔ | | | | | ↔ NK at 4 wks old | ↔ | ↓ liver wt ♀ at 4 wks but ↔ at 8 wks ↔ ♂. |
| | 1.0 | 17 | ~ 9 | ↔ | ↔ | ↔ | | | | | ↔ NK at 4 wks old<br>↓ NK ♂, ↔♀, at 8 wks old | ↔ | ↔ liver wt ♂♀ at 4 & 8 wks old |
| | 5.0 | 85 | ~ 50 | ↔ | ↔ | ↔ | ↓ B cell ♀ ↔ ♂ 4 wks old. | ↓ B cell ♀ ↔ ♂ 4 wks old. | | | | | ↑ liver wt ♂ ↓kid wt ♀ at 4 wks old. |
| | | | | | | | ↔ ♂♀ at 8 wks old | ↓ CD3+ CD4+ ♂ at 8 wks. | | | ↓ NK ♂ ♀ at 8 wks old | ↓ ♂ at 8 wk old ↔ ♀ | ↔ ♂♀ at 8 wks old. |

Study is an immunotoxicity developmental investigation. Pregnant dams gavaged daily on GD 1- 17. TAD calculated from mg/kg/d x 17 d.

Dam PFOS serum levels not measured. Values above are extrapolated from data in Lau *et al*. (2007).

Data is for 2 replicate experiments for pups at 4 or 8 weeks old.

Only maternal data provided is body weight; no significant weight loss (agrees with Thibodeaux *et al*. 2003a).

Pups evaluated for organ mass & cellularity, NK activity, PFC after sensitisation with SRBC 4d prior, splenic & thymic cell subpopulations.

Placental & lactational exposure.

Based on decreased activity of spleen NK cells to 51Cr-labelled Yac-1 cells from 8 week old male pups the LOEL dam dose = 1 mg/kg/d. At this dose female pups not affected and plaque forming assay no different to controls. NOEL = 0.1 mg/kg/d.

Lack of maternal serum concentrations, observations of dams, and wide dose spacing limit the usefulness of this study.

Although serum PFOS not measured in this study Lau *et al*. (2007) reports a maternal serum concentration at term of 9 mg/L after gavage dosing with 1 mg/kg/d for GD 1 – 17. It may be anticipated that serum PFOS concentrations at the LOEL in the Keil *et al*. (2008) study should also be around 9 mg/L, and at the NOEL about 1 mg/L given serum concentrations are proportional to dose (Thibodeaux *et al* 2003a, Lau *et al*. 2007). Contextual information is provided by the BMDL5 in mice for decreased maternal liver weight during pregnancy, pup survival and cleft palate of 1.31, 3.88 and 3.53 mg/kg/d respectively (Thibodeaux *et al* 2003a). Keil *et al*. (2008) indicate there was no significant weight loss in pregnant dams (data not provided).

# Appendix B2: Descriptions of selected PFOA animal studies

## DeWitt *et al*. (2016)

Experiment conducted to investigate if:

1. Suppression of TDAR is dependent on PPARα activation.

2. T- or B cells are targeted.

PFOA in drinking water for 15d.

*Assessing TDAR:* WT & PPARα female knock out mice.
Doses 0, 7.5 & 30 mg/kg/d for 15 d.
On d11 intravenous injection of SRBC (T cell dependent antigen, IgM response).
5d alter serum SRBC specific IgM measured (this peak IgM titre).

*Assessing TIAR:* C57BL/6N female mice.
Doses 0, 0.94, 1.88, 3.75 & 7.5 mg/kg/d for 15 d.
On d11 intravenous DNP (Type 2 T-cell independent antigen).
7d later serum specific DNP IgM measured (delayed hypersensitivity).

*Immunophenotyping studies:* C57BL/6N female mice.
Doses 0, 3.75 & 7.5 mg/kg/d

(immunosuppressive but not toxic wrt decreased body, spleen or thymus weights).

Spleens harvested. At 10d cell phenotyping without immunisation. At 11d intravenous SRBC, phenotyping on d13 & d15.

### Findings

| | | Dose (mg/kg/d) | | | | |
|---|---|---|---|---|---|---|
| **Endpoint** | **Mouse strain** | **0.94** | **1.88** | **3.75** | **7.5** | **30** |
| Body weight | WT | | | | ↔ | ↓ |
| | KO | | | | ↔ | ↔ |
| Immune organs | WT | | | | ↓ Thymus | ↓ Spleen |
| | KO | | | | ↔ | ↔ |
| | C57BL/6N | | | | ↓ | ↓ |
| TDAR | WT | | | | ↔ | ↓ |
| | KO | | | | ↔ | ↓ |
| TIAR | C57BL/6N | ↔ | ↓ | ↓ | ↓ | ↓ |
| | | | | Reduced the same (10.3%, 9.3%,10.7%) | | |
| Splenocyte phenotypes | C57BL/6NNo immunisation | | | ↔ | ↔ | ↔ |
| | 3 & 5d after immunisation | | | changed a | changed a | |
| | | | | changed a | ↔ | |

*An empty cell indicates endpoint not assessed.*
*a Changes in CD4/CD8 +/- were different depending on days post immunisation.*
*↓ NK cells only at 3.75 mg/kg/d and at 5d post immunisation.*

**Author's conclusions**

- Suppression of TDAR independent of PPARα.
- Suppression of TDAR & TIAR with minimal sub-population effects suggests effects mediated by disruption of B-cell/plasma cell function.

Tentative NOEL 0.94 mg/kg/d for ↓ TIAR at 1.88 mg/kg/d (LOEL, note no dose response, see Section 6 for discussion).

## DeWitt *et al*. (2008)

Evaluated humoral (adaptive) modulation by PFOA after i.v. immunisation with SRBC and

- Measuring 5d later, serum SRBC-specific IgM (initial response).
- Two weeks after immunisation challenged with i.v. SRBC, 5d later serum SRBC-specific IgG measured (memory response).
- Delayed type hypersensitivity assessed with bovine serum albumin (BSA) s.c. in Freud's complete adjuvant as immunising agent, after 7d challenged with BSA footpad injection and measuring oedema response (foot pad thickness).

*Recovery experiment:* C57BL/6J mice (same as Yang *et al*. 2000, 2001, 2002a)
Daily gavage in water for 15d.
30 mg/kg/d (similar to Yang *et al*. 2000, 2001, 2002).
50% of mice dosed for 10d, rest for 15d.

| | Serum PFOA (mg/L) | |
|---|---|---|
| **30 mg/kg/d for** | **1d post dosing** | **15d post dosing** |
| 10d | 84.7 ± 9.8 | 47.8 ± 2.1 |
| 15d | 266.5 ± 23 | 68.0 ± 3.8 |

*Dose response experiment:* C57BL/6 female mice.
Dosed in drinking water for 15 days.
Expt I: 0, 3.75, 7.5, 15 & 30 mg/kg/d.
Expt II: 0, 0.94, 1.88, 3.75 & 7.5 mg/kg/d.

| Dose mg/kg/d | Serum conc [a] (mg/L) | Body weight | Spleen weight | Thymus weight | SRBC - IgM | SRBC - IgG | DHT | Liver weight |
|---|---|---|---|---|---|---|---|---|
| 0 | 0.05 ± 0.005 | | | | | | | |
| 0.94 | ND | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↑ |
| 1.88 | ND | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↑ |
| 3.75 | 74.9 ± 2.7 | ↔ | ↓? | ↔ | ↓ | ↑ | ↔ | ↑ |
| 7.5 | 87.2 ± 3.3 | ↔ | ↓? | ↔ | ↓ | ↑ | ↔ | ↑ |
| 15 | 128.1 ± 6.8 | ↔ | ↓ | ↓ | ↓ | ↔ | ↔ | ↑ |
| 30 | 163.6 ± 8.4 | ↓ | ↓ | ↓ | ↓ | ↔ | ↔ | ↑ |

*ND = Not Determined.*
*a Serum PFOA concentration 1 day post dosing.*

Reductions in antibody titres occurred at lower doses than those causing decreased immune tissue weights.

- The ↑ in IgG is speculated by authors to represent progressive but not complete recovery or rebound of IgG synthesis.
- There are methodological differences in this study compared to Yang *et al*. (2002b). Yang *et al*. (2002b) used horse RBC and assessed PFC and HRBC-specific IgM and IgG in unimmunised animals. IgG was only assessed after a single immunisation and at the same time as HRBC-specific IgM, not after a booster immunisation.

$BMD_{1SD}$ = 3.06 mg/kg/d ($BMDL_{1SD}$ 1.75 mg/kg/d).

NOEL = 1.88 mg/kg/d for ↓ circulating SRBC specific IgM after inoculation.

LOEL = 3.75 mg/kg/d (Serum PFOA 74.9 ± 2.7 mg/L, 50 – 100x greater than population near PFOA factory).

## DeWitt *et al*. (2009c)

Study addresses hypothesis that the observed immunosuppression is secondary to elevated serum corticosterone levels by assessing immune function in adrenalectomised (adx) or sham-operated C57BL/6N female mice.

PFOA doses: Female C57BL/6 mice 0, 3.75, 7.5, or 15 mg/kg/d in drinking water for 10 days.

Immune tests: TDAR, ie. primary antibody response to T-cell dependent antigen (SRBC).
Day after exposure ended, i.v. SRBC with SRBC-specific IgM measurement 5d later.

General tests: Body weight only (no weight reported for immune tissue or liver).
Serum clinical chemistry, including corticosterone.
No serum PFOA measurements.
No liver histology, relied on serum enzymes as indicator of hepatic toxicity.

**Findings**

Sham animals, ↓ TG at all doses at 5d, ↔ at 11d; all other clinical chemistry ↔.
↓ body wt at 15 mg/kg (recovered 5d later), ↑↑ corticosterone.
↓ SRBC-specific IgM at 15 mg/kg (↔ at 7.5 mg/kg).

Adrenalectomised, ↓ TG at 7.5 & 15 mg/kg at 5d, ↔ at 11d; all other clinical chemistry ↔.
↓ body wt at 7.5 mg/kg (recovered 5d later) & 15mg/kg, sl↑ corticosterone.
↓ SRBC-specific IgM at 7.5 & 15 mg/kg.

**Conclusions**

No liver toxicity (as per serum enzymes) and failure of adrenalectomy to protect mice from the immunosuppressive effects of PFOA indicates that suppression of antibody synthesis is not the result of liver toxicity or stress-related corticosterone production.

NOEL = 7.5 mg/kg for 10d based on decreased TDAR.

## Yang *et al*. (2000)

This investigates a number of PPARα agonists (PFOA, DEHP, Wy-14 643, nafenopin) in male C57Bl/6 mice in diet. PFOA at 0.02% and animals evaluated at 2, 5, 7 & 10 days.

Mice ate 3g feed/d, ave body wt over 10 d was 22 g, therefore intake = ~27 mg PFOA/kg/d.

All PPARα agonists had similar effects (↓ body weight, ↓ spleen wt) and time course resembled that for increased liver weight and peroxisome proliferation (although not explicitly measured).

Decrease in body weight is due to very specific loss of adipose tissue.

PFOA for 7 days reduced total number of thymocytes and splenocytes (T- & B-cells).

Authors concluded thymic and splenic atrophy by PFOA were not immune direct effects but involved an indirect pathway.

Study not amenable for NOEL determination.

## Yang *et al*. (2001)

Similar design as Yang *et al*. (2000) but peroxisome proliferation measured. Increased liver weight and peroxisome proliferation occurred prior to decreases in thymus and spleen weight. However, in contrast to the persistence of the increase in liver weight and peroxisome proliferation after withdrawal of PFOA, rapid recovery of normal thymus and spleen weights and cell numbers was observed within 10 days.

Although immune tissue weights and cellularity assessed there were no immune function tests or serum PFOA measurements.

## Hu *et al*. (2010)

Examines the developmental toxicity of PFOA.

PFOA doses: C57BL/6 mice 0, 0.5, or 1 mg/kg in drinking water for GD 6 – 17, ♀ pups evaluated.

On PND 43 ♀ offspring i.v. SRBC immunisation SRBC-specific IgM in serum assessed 5d later and serum SRBC-specific IgG measured 5d after a booster immunisation 14d after the first (n = 8 pups from at least 2 litters for each of IgM & IgG).

### Findings

Litter weights ↓ (10%) at 1 mg/kg/d.
↔ liver & lymphoid organ weight in pups at time of organ collection.
↔ TDAR either as SRBC-specific IgM or SRBC-specific IgG.

Serum PFOA in offspring of dams dosed GD 6 -17 in drinking water

| Pup gender | Age (days) | Serum PFOA (mg/L) [a] | |
|---|---|---|---|
| | | 0.5 mg/kg/d | 1 mg/kg/d |
| Male | 20 | 1.56 | 3.41 |
| Female | 48 | 0.12 | 0.18 |
| Female | 63 | ~0.02 | ~0.055 |

*a Data are mean serum concentrations, some values are approximate as they are read from a graph.*

NOEL for developmental immunomodulation = 1 mg/kg/d.

(In pilot studies pups did not survive doses > 1mg/kg/d, survival ↓ 75% at 5 mg/kg/d).

## Loveless *et al* (2008)

♂ CD rats & CD-1 mice dosed by gavage with 0.3 – 30 mg/kg/d with IAPFO for 29 days.

### Evaluations

Body & immune organ weights, clinical chemistry measured.

Histopathology of liver, spleen, thymus, lymph nodes, bone marrow, femur/knee joint, sternum.

Serum corticosterone.

TDAR (SRBC-specific IgM after i.v. inoculating with SRBC 5 or 6 days before sacrifice).

| Dose mg/kg/d | CD Rat | | | | | | CD-1 Mice | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Body wt | Spl'n wt a | Liver wt | Cholb | Cort c | TDAR | Body wt | Spl'n wt | Liver wt | Chol | Cort | TDAR |
| 0 | | | | | | | | | | | | |
| 0.3 | ↔ | ↔ | ↔ | ↓ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ |
| 1 | ↔ | ↔ | ↑ | ↓ | ↔ | ↔ | ↔ | ↔ | ↑ | ↔ | ↔ | ↔ |
| 10 d | ↓ | ↔ | ↑ | ↔ | ↔ | ↔ | ↓ | ↓ | ↑ | ↓ | ↑ | ↓ |
| 30 | ↓↓ | ↔ | ↑ | ↔ | sl↑ | ↔ | ↓ | ↓ | ↑ | ↓ | ↔ | ↓ |

*a Spleen weight, b Cholesterol, c Corticosterone.*
*d NOEL for immune related endpoint (↓ TDAR) in CD-1 mice.*

- No immune-related changes occurred in rats, even at doses causing systemic toxicity (marked decrease in body weight gain).
- In mice, immune-related changes occurred only at doses causing significant and profound systemic toxicity (decrease body weight) and stress (increased corticosterone).

NOEL for immunological endpoints (mice) = 1 mg/kg/d.

LOEL for hepatic and immunological endpoints (mice) = 0.3 and 10 mg/kg/d respectively.

Authors concluded immune-related effects in mice are likely secondary to systemic toxicity and stress observed at high doses. These effects are decreased TDAR, decreased spleen and thymus weights and cell numbers; microscopic depletion/atrophy of lymphoid tissue.

This paper prompted DeWitt *et al*. (2009c) to investigate the dependence of PFOA immunomodulatory effects on toxicological stress. They used low doses not associated with gross toxicity and found suppression of antibody synthesis (TDAR) in C57BL/6N mice is not the result of liver toxicity or stress-related corticosterone production.

The difference in NOELs between Loveless *et al* (2008) and DeWitt *et al*. (2009c) is due to the wide dose spacing in Loveless *et al*. (2008), studies are not incompatible.

# Appendix C: Brief review of NTP (2016)

## Overview

In June 2016 the US Office of Health Assessment and Translation (OHAT), a division of the National Toxicology Program, released a draft systematic review of the published literature pertaining to immune system modulation by PFOS and/or PFOA (NTP 2016). Based on this review the NTP concluded that both PFOA and PFOS are presumed to be immune hazards to humans.

The pivotal outcome of the NTP (2016) review is hazard identification and classification, not identification of integrated NO(A)ELs or LO(A)ELs from the literature, or an assessment of immunotoxicological risk, or risk health effects that may result from altered immune function. The conclusion that PFOA and PFOS present an immune hazard to humans means at some level of exposure the function of the immune system may be changed. However the report does not address the issue for what level of exposure is immune function in humans likely to be compromised, as judged either by changed immune parameters or clinical outcome.

## Discussion

The overall objective of the NTP review was to develop hazard identification conclusions on the association between these PFASs and immunotoxicity. The systematic review, conducted according to the OHAT handbook (NTP 2015), summarises the extent of the literature and validity/bias of individual studies using a codified system for various aspects of information evaluation. For example, risk of bias in a particular study is rated as 'definitely low', 'probably low', 'probably high' or 'definitely high'. Animal and human studies are rated according to the level of confidence OHAT placed on the findings; (1) High, (2) Moderate, (3) Low, or (4) Very Low/No Evidence Available. The ratings are then combined using a narrative approach into five possible hazard identification conclusions: (1) Known, (2) Presumed, (3) Suspected, (4) Not classifiable, or (5) Not identified to be an immune hazard to humans. Due to heterogeneity of studies and small numbers of reliable studies across primary endpoints of immunosuppression (modulation of antibody response, disease resistance), hypersensitivity, and autoimmunity, meta-analysis was not conducted. Confidence in the body of evidence for the hazard classification is rated as high, moderate, low or very low. Each of the various ratings above has prescribed sets of criteria to assist with the rating binning. The NTP (2016) report also describes the limitations of the systematic review and the evidence base, it identifies data gaps and key research needs.

The conclusions were:

1. PFOA is presumed to be an immune hazard to humans based on:

    - a "high level of evidence" that PFOA suppressed the antibody response from animal studies and a moderate "level of evidence" from studies in humans, and
    - "a high level of evidence" that PFOA increased hypersensitivity-related outcomes from animal studies and "low level of evidence" from studies in humans

2. PFOS is presumed to be an immune hazard to humans based on

    - a "high level of evidence" that PFOS suppressed the antibody response from animal studies and
    - a "moderate level of evidence" from studies in humans that higher serum levels of PFOS are associated with suppression of antibody response.

The foundation of these conclusions is further discussed below. For both PFOA and PFOS the majority of data on primary health outcomes were for antibody responses.

High confidence was assigned to the results from animal studies but NTP (2016) had serious concern for risk of bias in the studies, nevertheless this was countered by demonstration of dose response. However while dose response was demonstrable in the studies, NTP did not comment or consider in the hazard analysis the very different dose response potency between studies.

As with PFOA high confidence for immune suppression was assigned to the PFOS animal data but again there was serious concern for risk of bias which was offset by evidence of dose-response. As with PFOA dose response potency differences between studies with PFOS (see Table 3.1 in this report) were not considered in the hazard classification reached by NTP (2016).

For both PFOA and PFOS, NTP acknowledges variability in the human studies with respect to the association between higher serum concentrations and lower antibody responses to some vaccines but not others within a study and between studies. This does not appear to have influenced the hazard ratings as the criteria adopted was for at least one measured immune parameter to be lower. It was indicated heterogeneity in the findings may be explained by variation between studies in the different vaccinations tested, time between vaccination and measurement of the antibody response, and analyses or ways to measure the antibody response. But there is no definitive explanation, only these possibilities.

NTP also discusses the possibility of co-founders, primarily other PFASs, on the reported associations in epidemiology studies as part of the risk of bias analysis. Issues with some, but not all, bioaccumulative substances showing the same associations, as discussed in Section 6 in this report, were downgraded due to NTP's acceptance of adjustments in various (complex) models accounting for the co-exposures specifically considered. NTP (2016) acknowledges the human studies have limited ability to differentiate effects of PFOA or PFOS from other PFASs, but consider the other PFASs to be effect modifiers rather than true confounders. Despite the concerns for risk of bias no epidemiology study was excluded on this basis.

Some issues discussed in NTP (2016) are:

- It is stated there is no *a priori* reason to suspect a specific window of susceptibility for PFOA or PFOS exposure to affect the antibody response (ie. developmental, childhood, or adult). NTP (2016) discusses associations are more consistent with PFAS measurements at the life stage when immune response was assessed rather than with maternal levels.
- Given the number of different analyses in some studies NTP (2016) indicates chance cannot be ruled out for some of the reported associations.
- In addition, it is well established that antibody levels decrease substantially in the months and years following vaccination. Thus there is a greater decrease in antibody level with more time between vaccination and the measurement of the antibody response. This increased time also allows for greater accumulation of PFOA or PFOS, hence the possibility of 'reverse causation' in some studies.
- Although multiple studies report higher PFOA and PFOS serum concentrations were associated with lower antibody levels, none of the studies clearly demonstrated an increase in the effect (ie. greater reduction in the antibody level or reduced rise in antibody level following vaccination) with higher exposure levels of PFOA or PFOS.
- There is low confidence in the body of evidence for human studies for PFOA or PFOS associations with infectious disease. Thus the lower antibody response to antigens has not, or has not yet been demonstrated to be translated into increased incidence of infectious disease.

With regard to hypersensitivity:

- For PFOA it was concluded there was low confidence that PFOA exposure during childhood is associated with increased hypersensitivity responses.
- High confidence was assigned to PFOA increasing hypersensitivity in animal studies. This however is only based on two studies (Fairley *et al*. 2007, Ryu *et al*. 2014). There are aspects of these studies that do not appear to have been incorporated into the NTP (2016) scoring system. Ryu *et al* (2014)[28] evaluated lung function after feeding pregnant mice and offspring a diet with low concentration (0.0004%) of PFOA or PFOS. Offspring were sensitised to ovalbumin and then challenged with methacholine or ovalbumin. At the time of challenge PFOA serum concentrations were high (4.8 mg/L), PFOS was not measured. PFOA but not PFOS increased lung responses, ie. increased sensitivity, to methacholine but not to ovalbumin challenge. The response to methacholine is not immune mediated; its action is mediated via muscarinic M3 receptors. Total or ovalbumin specific IgE antibodies were not measured, and neither treatment altered the magnitude or severity of inflammation in the airway hyper-responsiveness. The Fairley *et al* (2007) study is unusual in that different strength solutions were applied to the ears of mice. Despite the fact that PFOA is poorly absorbed through the skin (Fasano *et al*. 2005) there was a dose response increase in hypersensitivity response to ovalbumin in sensitised mice, including increased OVA-specific IgE. Serum concentrations of PFOA were not measured.
- For PFOS the conclusion from several cross-sectional studies was “very low confidence” that exposure to PFOS during childhood is associated with changes in the hypersensitivity responses in children. Also for animals NTP (2016) has low confidence that exposure to PFOS is associated with a change in the hypersensitivity response because the results are inconsistent from a single study that directly tested airway hypersensitivity and a second study that examined antigen-specific IgE in mice (Dong *et al*. 2011, Ryu *et al*. 2014).

28 Ryu *et al*. (2014) exposed pregnant mice and the offspring to a single level of PFOA or PFOS in the diet. Offspring were assessed for lung function at 12 weeks of age; prior to assessment mice were sensitised to ovalbumin adsorbed onto alum using 2 i.p. injections. After sensitisation mice were challenged with either nebulised methacholine or application of ovalbumin onto nostrils. Serum PFOA concentration in offspring at 12 weeks of age was 4.8 ± 1.1 mg/L, PFOS serum levels were not measured.

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