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Review article

The derivation of a Reference Dose (RfD) for perfluorooctane sulfonate (PFOS) based on immune suppression

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ABSTRACT

Exposure to perfluorooctane sulfonate (PFOS) is ubiquitous in populations and environments worldwide. Its long half-life in humans, indefinite persistence in the environment, and awareness of its widespread presence in drinking water make the human health assessment of PFOS a priority. While developmental, endocrine, and hepatic effects, and increased serum cholesterol are among the outcomes resulting from PFOS exposure, immunosuppression has also consistently emerged as an adverse effect. An in-depth review of the relevant scientific literature on the toxicology of PFOS has identified immunosuppression as a sensitive endpoint for PFOS toxicity. Here, we focus specifically on that endpoint and provide a detailed derivation of a Reference Dose (RfD) of 1.8×10^{-6} mg/kg/day for chronic human exposure to PFOS. This RfD is based on decreased plaque-forming cell (PFC) response in mice, an endpoint that reflects suppression of the immune response to a foreign antigen. We additionally identify two endpoints in the epidemiology literature, decreased vaccine response and increased incidence of childhood infections, that are associated with PFOS exposure and that are consistent with and support the decreased PFC response endpoint from animal studies. We provide a weight of evidence analysis integrating the evidence from animal and epidemiology endpoints. Finally, we compare this RfD to the PFOS RfD derived by the United States Environmental Protection Agency (USEPA) Office of Water based on a developmental endpoint. Based on this comparison, and given our assessment, the USEPA RfD does not provide sufficient protection against the adverse health effects of PFOS. The RfD derived herein is intended to be public health protective and appropriately minimizes PFOS exposure based on available evidence.

1. Introduction

Perfluorooctane sulfonate (PFOS) is a widely occurring environmental contaminant of public health concern. The chemistry of PFOS (e.g., its carbon-fluorine bonds) led to its use in a wide array of commercial and industrial applications, such as a stain/water repellant for fabrics, in metal plating and finishing, photograph development, and food packaging (USEPA, 2016a). Notably, PFOS also has been a constituent of aqueous film forming foam (AFFF) used in extinguishing Class B fires (i.e., involving flammable liquids) (Seow, 2013). Although the production and use of PFOS and related chemistries (e.g., perfluorooctanesulfonyl fluoride) was phased out in the United States in 2002 (USEPA, 2016a), the persistence of PFOS (see below) results in its continuing presence in the environment. PFOS is detected in ground and surface water, fish and other biota, soil, and house dust both near sources of contamination and globally (USEPA, 2016a). PFOS has been found in drinking water at numerous locations throughout the U.S. and worldwide, particularly near sites where AFFF was used (Hu et al., 2016; Post et al., 2017).

The chemistry of PFOS also has important implications for its distribution in environmental media and biota. Due to its resistance to physical and biological degradation, PFOS persists indefinitely in the environment. PFOS has both hydrophilic and lipophilic characteristics, and strongly, but non-covalently binds to protein, including in fish (Conder et al., 2008), while not accumulating in lipid-rich tissues.

The presence of PFOS and its precursors in multiple environmental

Abbreviations: BMD, benchmark dose; BMDL, benchmark dose-lower confidence limit; BMDS, USEPA Benchmark Dose Software; C8, synonym for perfluorooctanoic acid (PFOA); CI, confidence interval; CL, clearance factor; DWQI, New Jersey Drinking Water Quality Institute; IRR, incidence rate ratio; LOAEL, Lowest-Observed-Adverse-Effect Level; NHANES, National Health and Nutrition Examination Survey; NOAEL, No-Observed-Adverse-Effect Level; NTP, National Toxicology Program; OR, odds ratio; PFC, plaque forming cell; PFAS, per- and polyfluoroalkyl substances; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; POD, point of departure; PPAR, peroxisome proliferator-activated receptor, RfD, reference dose; SD, standard deviation; UF, uncertainty factor; USEPA, United States Environmental Protection Agency

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Table 1
Comparison of studies assessing effects of PFOS on PFC response in mice.

Study	Strain/ sex/ age	PFOS cation used	Duration and route of exposure	Animals per dose group	Administered PFOS Dose (mg/kg/day)	Terminal Serum [PFOS] (ng/ml) [a]		PFC response in animals (per 10^6 splenocytes)	
Dong et al. (2009)	C57BL/6 Male Adult (8–10 weeks)	K^+	60 days Gavage	10	0	48		597 [b]	
					8×10^{-3}	674		538 [b]	
					0.08	**7,132** [a]		416 [b]	
					0.42	21,638		309 [b]	
					0.83	65, 426		253 [b]	
					2.1	120,670		137 [b]	
Peden-Adams et al. (2008)	B6C3F1 Male and Female Adults (7–8 weeks)	K^+	28 days Gavage	5/sex		Male	Female	Male	Female
					0	12.1 [c]	16.8 [c]	~ 3,500 [d]	~ 3,000 [d]
					1.7×10^{-4}	17.8 [c]	ND	~ 2,800 [d]	~ 2,600 [d]
					1.7×10^{-3}	**91.5** [a,c]	88.1 [c]	~ 1,400 [d]	~ 3,200 [d]
					3.3×10^{-3}	131 [c]	123 [c]	~ 1,100 [d]	~ 2,500 [d]
					0.02	ND	**666** [a,c]	~ 1,500 [d]	~ 1,500 [d]
					0.03	ND	ND	~ 1,600 [d]	~ 1,000 [d]
					0.17	NR	NR	~ 1,400 [d]	~ 800 [d]
Keil et al. (2008)	B6C3F1 Male and Female Challenged as adults (8 weeks)	K^+	GD 1–17 (Gestational exposure) Gavage	6/sex (1/litter)				Male	Female
					0.0	ND		~2,300 [d]	~2,300 [d]
					0.1	ND		~2,300 [d]	~2,300 [d]
					1.0	ND		~1,500 [d]	~2,250 [d]
					5.0 [a] (LOAEL M;NOAEL F)	ND		~1,000 [d]	~2,250 [d]
Zheng et al. (2009)	C57BL/6 Male Adults (8–10 weeks)	K^+	7 days Gavage	12	0	≤ 50 [e]		~ 3,700 [d]	
					5	**1.1×10^5** [a]		~ 1,400 [d]	
					20	2.8×10^5		~ 800 [d]	
					40	3.4×10^5		~ 500 [d]	
Qazi et al. (2010)	B6C3F1 Male Adults (7–8 weeks)	TEA	28 days Dietary	5	0	41		~ 7,800 [d]	
					0.25	1.2×10^4		~ 8,200 [d]	

F – Female; M – Male; ND – Not determined; NR – Not reported (exceeded calibration); PFC – Plaque forming cell; TEA – Tetraethylammonium
Note: All studies used the Cunningham and Szenberg (1968) modification of the Jerne and Nordin (1963) method for determining PFC response, except for Keil et al. (2008) which used the Jerne and Nordin (1963) method.
[a] Bolded number is LOAEL in ng/ml or in mg/kg/day if serum PFOS concentration not determined
[b] Presented in graphical form in Dong et al. (2009). Numerical data obtained from G-H Dong, personal communication May 2016
[c] Authors reported measured serum PFOS concentrations in ng/g and stated that this concentration is approximately equivalent to ng/ml
[d] Visually estimated from graphic presentation in respective studies
[e] Reported as below detection. Detection limit reported as 0.05 mg/L (50 ng/ml)

media allows for multiple sources of exposure by humans. In contrast to other well-known persistent and bioaccumulative compounds such as PCBs and dioxins, PFOS is water soluble and drinking water is an important exposure route. Additionally, infants may be exposed to PFOS through breast milk (ATSDR, 2018).

Since 1999, the National Health and Nutrition Examination Survey (NHANES) has measured PFOS in the serum of a representative sample of the U.S. general population. As of 2013–2014, the median and 95th percentile of serum PFOS concentrations were 5.2 and 18.5 μg/L, respectively (ng/ml; CDC, 2017). Although the level of PFOS in human serum has been declining, the human half-life of 5.4 years (Olsen et al., 2007) raises a particular concern for adverse health effects in humans.

Health hazard assessments of PFOS have generally identified increased serum cholesterol, liver effects, decreased thyroid hormone levels, immunotoxicity, and developmental effects such as offspring mortality, decreased body weight, and neurotoxicity. While human cancer data are inconsistent, liver tumors have been observed in rats (reviewed in ATSDR, 2018; USEPA, 2016b).

Quantitative assessments of identified health effects have developed daily oral intake values (ng/kg/day) intended to be protective for chronic exposure (e.g., Tolerable Daily Intake [TDI], Reference Dose [RfD]) to PFOS (Dong et al., 2017; Lilienthal et al., 2017). Over time, these values have trended lower (Dong et al., 2017). To date, the bases for the derivation of these values have primarily been decreased serum triiodothyronine (T3) levels in monkeys, liver effects in rats, or decreased offspring body weight in rats (reviewed in Dong et al., 2017). Although consistently identified as an effect of PFOS, immunotoxicity has not been used as the primary basis (i.e., the critical effect) for daily intake values despite strong evidence that this effect can result from exposure to low levels PFOS.

As part of an independent quantitative assessment of PFOS (DWQI, 2018), a comprehensive literature search and screening was conducted to identify relevant human and laboratory animal information for the identification of potential health hazards from PFOS exposure. Immune suppression was selected as the critical effect and the basis for quantitative risk assessment. Specifically, the immunotoxic effect selected for the RfD was decreased plaque forming cell (PFC) response (Jerne and Nordin, 1963; Cunningham and Szenberg, 1968), in mice following inoculation with a foreign antigen (sheep red blood cells [SRBCs]) as reported in Dong et al. (2009). Here, we focus and expand on the qualitative (Hazard Identification) and quantitative (Exposure-Response) rationale for using immune suppression from Dong et al. (2009) as the basis for development of an RfD for PFOS.

2. Hazard identification

2.1. Strategy for identification of immune suppression as the critical effect for PFOS

The full Hazard Identification process, including the criteria for identification of relevant human and animal studies from the scientific literature, is detailed in the New Jersey Drinking Water Quality Institute (DWQI, 2018) PFOS assessment. Briefly, developmental, endocrine, hepatic, and immune toxicity were among the potential outcomes identified from PFOS exposure (DWQI, 2018). As described in the DWQI (2018) document, the epidemiology data were not suitable

for dose-response assessment although they provide important support for the overall hazard identification. Therefore, the RfD presented in the DWQI (2018) document is based on animal data. The selection of candidate critical effects was based on comparing Lowest-Observed-Adverse-Effect Levels (LOAELs) reported as serum PFOS concentrations. As discussed in the Exposure-Response section, the internal dose metric of serum PFOS concentration is preferable to external administered dose (e.g., mg PFOS/kg body weight/day) for use in risk assessment.

In comparing the most sensitive candidate critical effects (i.e., with serum PFOS LOAELs < 10,000 ng/ml) from studies appropriate for RfD development (e.g., duration of at least 30 days), several hepatic effects, endocrine effects in adults and offspring, immune system effects, and developmental effects (e.g., increased mortality) were identified. Based on the timing of endpoint ascertainment relative to serum PFOS analysis, biological significance, and suitability for dose-response analysis in the DWQI (2018) document, decreased PFC response as reported by Dong et al. (2009) was identified as the most sensitive of the candidate critical effects. Herein, we present the detailed assessment of the RfD based on this immunosuppression endpoint including supporting animal and epidemiology studies.

2.2. Animal studies assessing plaque forming cell response

Criteria (e.g., > 30 days exposure duration) applied in the Hazard Identification process (DWQI, 2018) ultimately led to the identification of Dong et al. (2009) as a candidate principal study for RfD development. In addition, four other studies, all in mice, examining the PFC response endpoint that did not meet the initial selection criteria (i.e., study duration) were also identified. We address these studies here from the standpoint of assessing the consistency of the PFC response. Table 1 summarizes salient methodological information and results for these five studies.

2.2.1. Dong et al. (2009)

Dong et al. (2009) exposed adult male C57BL/6 mice to PFOS via oral gavage for 60 days at administered doses of 0, 0.008, 0.08, 0.42, 0.83, and 2.1 mg/kg/day. There was a continuous and monotonic decrease in PFC response that reached statistical significance compared to controls at 0.08 mg/kg/day (7132 ng/ml in serum; the LOAEL) and was statistically significant for trend. The NOAEL was thus 0.008 mg/kg/day (674 ng/ml in serum) (Fig. 1). Other endpoints identified in this study are described in Appendix A.

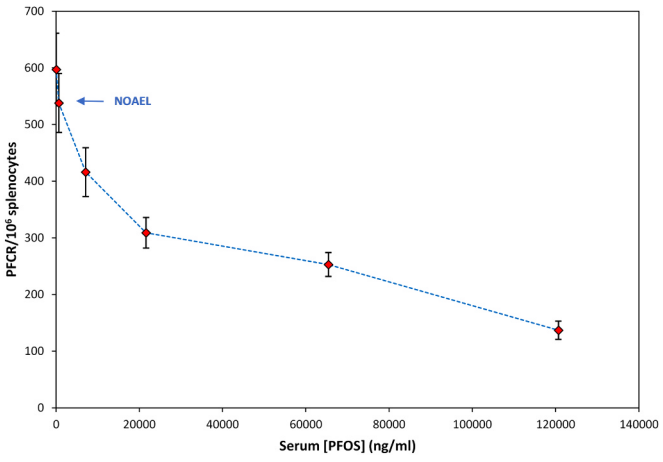

Fig. 1. Dose-response data for PFC response (PFCR) as adapted from Dong et al. (2009). Data are presented as mean ± standard error of the mean. PFC response data were presented in graphical form in the publication; numerical data were obtained by personal communication with author.

2.2.2. Keil et al. (2008)

Keil et al. (2008) demonstrated that developmental PFOS exposure *in utero* and through lactation via maternal dosing during gestation results in immunosuppression in adult male offspring (LOAEL 5.0 mg/kg/day). However, Keil et al. (2008) did not measure serum PFOS concentrations associated with each dose group. Because serum concentrations are preferable to administered doses as the dose-metric for PFOS (see Exposure-Response section), Keil et al. (2008) is not appropriate for dose-response analysis.

2.2.3. Peden-Adams et al. (2008)

Peden-Adams et al. (2008) did not meet the criterion of exposure duration of > 30 days for RfD development, and was, therefore, not included in the initial selection of studies considered for dose-response analysis. This was the only study that assessed PFC response in female as well as male mice following PFOS exposure as adults. This study reported decreased PFC response in male and female B6C3F1 mice following 28 days of PFOS exposure. As summarized in Table 1, the male and female LOAELs (91.5 and 666 ng/ml, respectively) were the most sensitive among all the PFC response studies identified. Given that the LOAEL was below that from the longer Dong et al. (2009) study, we analyzed the dose-response in Peden-Adams et al. (2008).

2.2.4. Zheng et al. (2009)

While Zheng et al. (2009) reported decreased PFC response following only 7 days of exposure, administered PFOS doses ranged from 5 to 40 mg/kg/day, resulting in serum PFOS concentrations > 100,000 ng/ml. These serum PFOS concentrations were at least two orders of magnitude higher than serum PFOS concentration-based LOAELs in Peden-Adams et al. (2008) and Dong et al. (2009). Due to its much higher LOAEL, Zheng et al. (2009) was not considered to be appropriate for dose-response analysis.

2.2.5. Qazi et al. (2010)

Although it shares some design similarities with Peden-Adams et al. (2008), Qazi et al. (2010) did not observe a decrease in PFC response following PFOS exposure. Whereas, both Qazi et al. (2010) and Peden-Adams et al. (2008) assessed PFC response in B6C3F1 male mice exposed to PFOS for 28 days, the serum PFOS concentration at the NOAEL (12,000 ng/ml) in Qazi et al. (2010) was more than 90 times the highest serum PFOS concentration (131 ng/ml) reported to cause decreased PFC response in males in Peden-Adams et al. (2008). The inconsistency in results between these studies may be due to the dietary route of exposure in Qazi et al. (2010) compared to the gavage exposure in Peden-Adams et al. (2008), the use of a different PFOS salt (i.e., tetraethylammonium in Qazi et al., 2010 versus potassium in Peden-Adams et al., 2008), and/or non-study-design factors (e.g., possible differences in SRBC sources, animal handling and husbandry). The use of a single dose group (plus control) makes this study inappropriate for dose-response analysis. In addition, the existence of four positive studies makes this lone negative study an outlier.

2.3. Selection of Dong et al. (2009) as the principal study

Given the foregoing considerations, Dong et al. (2009) and Peden-Adams et al. (2008) were the two studies of the effect of PFOS on PFC response considered for dose-response analysis. Although Peden-Adams et al. (2008) provides the most sensitive observation of decreased PFC response, we judged the Dong et al. (2009) study to be the more appropriate basis for the development of an RfD. This decision is based on the extreme sensitivity of the dose-response in the Peden-Adams et al. (2008) study compared to the other positive studies (as well as other considerations discussed below). Specifically, it is noted that the serum concentration at the NOAEL in the Dong et al. (2009) study (674 ng/ml) was higher than the maximum reported serum concentrations (131 ng/ml for males; 666 ng/ml for females) in Peden-Adams et al. (2008). In

Table 2
Summary of epidemiological studies investigating the association between PFOS exposure and antibody response.

Age of population at vaccine antibody measurement (n)	Central tendency serum PFOS concentration [a] (age at serum PFOS measurement)	Outcome by vaccine type [b] Percent change in antibody (95% CI)					
		Tetanus	Diphtheria	Rubella	Measles	Influenza [c]	Mumps
Grandjean et al. (2012) (longitudinal birth cohort)							
5 yrs old	27.3 ng/ml (maternal)	X [d]	↓	— [e]	—	—	—
Pre-booster (509 to 537)	16.7 ng/ml (5 yrs old)	pre-booster	39% [f] (-54.7, -16.9) pre-booster				
Post-booster (419 to 440)		↓ 29% [f] (-45.5, -6.1) post-booster	21% [g, f] (-37.5, 0.9) post-booster				
7 yrs old		X	↓	—	—	—	—
Post-booster (408)			28% [f] (-45.8, -3.3)				
Grandjean et al. (2017) (longitudinal birth cohort)							
5 yrs old	20.6 ng/ml [h] (maternal at birth)	X	X [i]	—	—	—	—
Pre-booster at 18 mos (275)	7.1 ng/ml (18 mos. old)		↓				
at 5 yrs (349)	4.7 ng/ml (5 yrs old)		24% [f, j] (-36.9, -9.6)				
Granum et al. (2013) (prospective birth cohort)							
3 yrs old (49–51)	5.6 ng/ml (maternal)	X	—	↓ 8% [k] (-14, -2)	X	X	—
Stein et al. (2016) (cross-sectional)							
12–19 yrs old (1,186-1,190)	20.8 ng/ml (≥ 12 yrs old)	—	—	↓ 13% [f, l] (-19.9, -6.2)	X	—	↓ 6% [f, l] (-9.9, -1.6)
Kielsen et al. (2016) (convenience sample)							
Adults (mean 37.9 yrs old) (12)	9.52 ng/ml (mean 37.9 yrs old)	X	↓ 12% [f] (-21.9, -0.3)	—	—	—	—
Looker et al. (2014) (prospective cohort)							
Adults (> 18 yrs old) (403)	8.32 ng/ml (> 18 yrs old)	—	—	—	—	X	—

(Note: Where both statistically significant associations and lack of associations for decreased antibodies with PFOS were reported for the same antibody under different ages of ascertainment and/or vaccine follow-up, details are reported here only for significant associations. The reader is referred to the original studies for full details.)

[a] Reported as median, mean, or geometric mean
[b] The increase in serum PFOS concentration for each change in specific antibodies is specified by a footnote
[c] For Granum et al. (2013), influenza B (Hib); for Looker et al. (2014), A/H3N2, A/H1N1 and influenza B
[d] "X" Measured but no significant response observed
[e] "—" Not determined
[f] Decrease in antibody level for each doubling of PFOS concentration
[g] Value (21%) was borderline statistically significant (95% Confidence Interval, -37.5% to 0.9%)
[h] Personal communication with P. Grandjean, January 2018
[i] Not statistically significantly associated with PFOS for cohort 5
[j] Combined cohorts (3 and 5)
[k] Decrease in antibody level for each unit increase in PFOS concentration
[l] Seropositive only

addition, the Dong et al. (2009) study reported a LOAEL of 7132 ng/ml. This serum PFOS concentration was approximately 80 times greater than the serum PFOS LOAEL in Peden-Adams et al. (2008). However, it should also be noted that the lowest dose in Dong et al. (2009) was the NOAEL and it resulted in a PFOS serum concentration of 674 ng/ml, a value 7 times greater than the LOAEL serum concentration in Peden-Adams et al. (2008). Given these considerations we judge that the Peden-Adams et al. (2008) study may be an outlier of the PFC response dataset in terms of its dose-response. The lack of response at a relatively high serum PFOS concentration in Qazi et al. (2010), and the lack of response in Dong et al. (2009) at a serum PFOS concentration above the serum PFOS LOAEL in Peden-Adams et al. (2008), raise concerns for the use of the PFC response results in Peden-Adams et al. (2008) as the sole basis for deriving a toxicity factor. This is particularly the case, given the absence of any confirmatory studies at similar serum PFOS concentrations.

Furthermore, stress is known as a potential cause of decreased immune function (Kaminski et al., 2008). Stress can, therefore, confound the assessment of direct chemical immunotoxicity. Serum corticosterone level is a measure of stress. In the Dong et al. (2009) study, serum corticosterone was elevated only at the two highest doses, corresponding to serum PFOS concentrations approximately 10–18 times the serum PFOS concentration at the LOAEL dose for PFC response. These results suggest that the decrease in PFC response in Dong et al. (2009) was due to PFOS exposure, rather than a secondary response to stress. The serum PFOS concentrations corresponding to increased serum corticosterone in the Dong et al. (2009) study were 2–3 orders of magnitude larger than the highest reported serum PFOS concentrations for the male mice in the Peden-Adams et al. (2008) study. Thus, it seems unlikely that the greater sensitivity in PFC response in Peden-Adams et al. (2008) could have resulted from stress due to PFOS exposure. However, because Peden-Adams et al. (2008) did not measure serum corticosterone, the possibility of stress resulting from handling and husbandry cannot be ruled out.

In summary, PFOS has the potential to cause decreased antibody response to foreign antigens as expressed in a reduction of the PFC response. This effect was consistently observed in multiple studies, with the magnitude of the PFOS exposure-response varying across studies. Additionally, use of this effect as the basis for the RfD is supported by human data (discussed below). Given this body of data, it was concluded that decreased PFC response is an appropriate basis for the PFOS RfD. For the reasons discussed above, we judge Dong et al. (2009) to be both a scientifically sound and a reasonably sensitive measure of this effect, and to be the most appropriate study for dose-response analysis

for the derivation of a PFOS RfD based on immunosuppression.

2.4. Mode of action considerations for PFC response in mice

The mode(s) of action responsible for a decrease in PFC response from PFOS exposure are not clear. As reviewed by Dewitt et al., (2009, 2012) and Corsini et al. (2014), it appears that the peroxisome proliferator activated receptor-α (PPARα) does not play a primary role in the PFC response effect of PFOS. This is particularly the case given the demonstration that PFC response did not differ between PPARα-null mice and wild-type (WT) mice exposed to PFOS as reported in Corsini et al. (2014). There is some suggestion of a secondary role for PPARα given the observation of a greater decrease in thymus weight (but not spleen weight) in WT versus PPARα-null mice (Qazi et al., 2009). Alteration in cell signaling has also been suggested as a possible mechanism (Corsini et al., 2014) given the absence of a reduction in the number of immune-relevant leukocyte populations accompanying a decrease in PFC response. Furthermore, whatever, the possible involvement of PPARα in immunotoxicity, PPARα does not play a significant role in PFOS-mediated hepatotoxicity, including hepatic tumors (DWQI, 2018).

2.5. Epidemiological evidence

Epidemiological evidence supports the use of decreased PFC response in animal studies as the basis for the PFOS RfD. There are two lines of evidence linking PFOS exposure to adverse immune effects, studies of vaccination response, and studies of the occurrence of childhood infections.

2.5.1. PFOS exposure and decreased vaccine response

We identified six studies addressing the association between vaccine response and PFOS exposure. Importantly, each of these studies investigated a population whose level of exposure to PFOS resulted from incidental exposure rather than from a specific source of contamination. Thus, these studies relate to levels of exposure to PFOS prevalent in the general population. Table 2 summarizes the structure, findings and strength of association of these studies.

2.5.1.1. Grandjean et al. (2012). This study of a birth cohort in the Faroe Islands provides support for the association of PFOS exposure and decreased vaccine response. Specifically, prenatal exposures (measured as maternal serum PFOS levels during pregnancy) were associated with decreased levels of diphtheria antibodies, and childhood exposure (serum PFOS levels at age 5) were associated with decreases of both diphtheria and tetanus antibodies (See Table 2 for details). It is particularly notable that the effect of prenatal PFOS exposure appears to have persisted until at least 7 years of age.

2.5.1.2. Grandjean et al. (2017). This study evaluated a more recent birth cohort (cohort 5) from the same location which had lower PFOS exposure than the earlier cohort evaluated by Grandjean et al. (2012; designated as cohort 3). Grandjean et al. (2017) did not show significant associations between maternal, 18-month old, or 5 years old serum PFOS concentrations and decreases in either tetanus or diphtheria vaccine antibodies at 5 years old. An additional analysis combining cohorts 3 and 5 ($n = 900$; Grandjean, P., personal communication; January 2018) showed a decrease (24.5%, $p = 0.002$) in diphtheria vaccine antibody that was significantly associated with a doubling of maternal serum PFOS concentration at birth (Grandjean et al., 2017). The combination of decreased exposure and decreased sample size in the newer cohort may have been responsible for the inability to identify a significant association between PFOS exposure and decreased vaccine response in the later cohort.

2.5.1.3. Granum et al. (2013). This study, nested in a Norwegian birth cohort, has the lowest serum PFOS concentration of the studies that evaluated vaccine response. The mean maternal post-partum serum PFOS concentration was 5.6 ng/ml, corresponding to between the median and 75th percentile exposure among U.S. adult females in the 2013–2014 NHANES database (CDC, 2017). A significant decrease in rubella antibody was observed (See Table 2 for details). As with the Grandjean et al. (2012) study, the Granum et al. (2013) study provides support for a prenatal effect of PFOS on decreased vaccine response.

2.5.1.4. Stein et al. (2016). Although providing further support for an effect of PFOS on decreased vaccine response, Stein et al. (2016) is a cross-sectional study using data from the 1999–2000 and 2003–2004 NHANES. It therefore, has the inherent weakness of all cross-sectional studies: the difficulty in inferring causality given the lack of information about the onset of the effect relative to the time course of exposure. However, this study has the advantages of a large sample size ($n \approx 1200$) and a U.S. study population rather than a European population as in the other vaccine studies. While the Grandjean et al. (2012, 2017) and Granum et al. (2013) studies provided evidence of an association between vaccine antibody levels and PFOS exposure in young children ($\leq$ 7 years old), it is notable that Stein et al. (2016) showed an association between PFOS exposure and vaccine (rubella) response in adolescents (12–19 years old) (See Table 2 for details).

2.5.1.5. Kielsen et al. (2016). This is a study in a convenience sample (i.e., a non-random sample collected based on a participant's proximity to the research), that is notable for showing a PFOS-vaccine antibody (diphtheria) association among adults. As a small study ($n = 12$), the ability to detect a significant response points to the consistency of this effect (See Table 2 for details).

2.5.1.6. Looker et al. (2014). This is a study in an adult population originating in the C8 cohort in Ohio and West Virginia. Among the studies in Table 2, Looker et al. (2014) is the exception in finding no statistically significant associations between PFOS exposure and decreased vaccine antibody levels. However, the Looker et al. (2014) study investigated a single vaccine, influenza, and the only other study to investigate influenza vaccine response, Granum et al. (2013), also failed to find an association for that vaccine despite finding an association for the rubella vaccine. Since different vaccines (and different preparations of the same vaccine) can differ in their inherent antigenicity, it may be that the influenza vaccine is less subject to the titer-reducing effects of PFOS than those vaccines that yielded significant associations.

2.5.2. Considerations for the interpretation of vaccine response studies

The six studies differ in the ages at which PFOS exposure and vaccine antibodies were measured, the time between inoculation and the measurement of antibody levels, the vaccine antibodies that were measured, the study populations, and the study designs. Nevertheless, the observation of an association of decreased vaccine antibodies with some measure of PFOS exposure for at least one vaccine antibody in all but one study supports an association between increased PFOS serum levels and decreased antibody response across different populations and different study designs.

In the studies in Table 2, PFOA, PFOS, and per- and polyfluoroalkyl substances (PFAS) overall, were generally tightly correlated as would be expected when exposures result from background sources that contain multiple PFAS. Although these studies reported on the associations between vaccine antibody levels and PFOS exposure as an independent variable, the extent of correlation among the PFAS meant that it was not possible to control for exposures to the other PFAS in the regression analyses for PFOS. Therefore, a weakness of the studies in Table 2 is that they cannot distinguish among an independent effect of PFOS, a generalized PFAS effect, or an effect due to specific PFAS other than

PFOS.

Although consistent associations were observed between significant reductions in the levels of some vaccine antibodies and increasing levels of PFOS exposure in these studies, vaccine antibody levels are known to be variable as a function of a number of factors including stress (Tournier et al., 2001) and time since vaccination (Martinón-Torres et al., 2017). Such variability is not necessarily a direct indicator of increased disease susceptibility as the otherwise healthy immune system has a reserve capacity (Chang et al., 2016). Therefore, it is particularly notable that in the Grandjean et al. (2012) study, being below the antibody level associated with clinical immune protection (0.1 IU/ml) was positively and significantly associated with serum PFOS concentration for diphtheria antibodies for the various age combinations of serum PFOS measurement and antibody levels (OR 1.60; 95% CI, 1.10, 2.34 to OR = 2.48; 95% CI, 1.55, 3.97). Similarly, a positive, but not significant, association was observed for being below the level of clinical immune protection and serum PFOS at 5 years old for post-booster tetanus antibodies (OR = 2.61; 95% CI, 0.77, 8.92). The other studies did not report assessments for a clinical benchmark (Granum et al., 2013; Stein et al., 2016; Kielsen et al., 2016; Looker et al., 2014).

2.5.3. PFOS exposure and infectious disease

Several studies examined possible associations between PFOS exposure and infectious disease as an indicator of a clinical effect of PFOS-mediated inhibition of immune response (Impinen et al., 2018; Goudarzi et al., 2017; Dalsager et al., 2016; Looker et al., 2014; Granum et al., 2013; Okada et al., 2012; Fei et al., 2010). While some of these studies also reported on additional endpoints, we focus here specifically on their findings regarding infectious disease. The results of these studies are summarized in Table 3.

2.5.3.1. Impinen et al. (2018). This study followed a cohort in Oslo, Norway (n = 641) through 10 years of age, and investigated associations between gestational PFAS exposure (via cord blood) and allergies, respiratory function and infectious outcomes. The mean serum PFOS concentration was 5.6 ng/ml (S.D. = 2.3 ng/ml), similar to the U.S. population as of 2013–2014 (CDC, 2017). PFOS (and PFOA) serum levels were positively and significantly associated with the number of parentally reported lower respiratory tract infections through 10 years of age. For a doubling of cord blood PFOS concentration, the regression model predicts a 50% increase in the number of lower respiratory infections.

2.5.3.2. Dalsager et al. (2016). In this longitudinal prospective study in the Odense (Denmark) Child Cohort, the median maternal pregnancy serum PFOS concentration was 8.07 ng/ml. When the children (n = 346) were between one and three years old, the mothers were prompted every two weeks to provide information on the number of days during the preceding period that the children had specific categories of health symptoms (fever, cough, nasal discharge, vomiting). For the continuous variable of the number of days with fever, the incidence rate ratio (IRR) for the highest versus lowest tertile of PFOS exposure was significantly elevated (1.65, 95% CI: 1.24, 2.18) and remained statistically significant following a Bonferroni correction for the several endpoints investigated. The trend across the tertiles of PFOS was also significant for the fever rate ratio. Similar results were observed for PFOA and, given the correlation between PFOS and PFOA serum levels, the authors did not attempt to control the analyses for the joint exposures.

2.5.3.3. Fei et al. (2010). This study followed the number of hospitalizations for infection in a large subset of the Danish National Birth Cohort (n = 1400 with 577 hospitalizations) from birth through an average of more than 8 years as a function of maternal prenatal serum PFOS concentration (mean = 5.6 ng/ml). Prenatal PFOS exposure was positively and significantly associated with the risk of hospitalization for infection for girls (but not for boys). For girls, the adjusted IRR for the third and fourth quartiles versus the 1st quartile of PFOS were 1.61 (95% CI: 1.05, 2.47) and 1.59 (95% CI: 1.02, 2.49) respectively, with a significant trend across quartiles (IRR 1.18, 95% CI: 1.03, 1.36). It should be noted that the data from this study only includes infections severe enough to warrant hospitalization.

2.5.3.4. Goudarzi et al. (2017). In this prospective, longitudinal cohort study in Japan, PFOS was measured in a blood sample supplied by pregnant women (n = 1558). The mean serum PFOS concentration was 5.46 ng/ml, similar to the that of the U.S. population (CDC, 2017). When children were four years old, the mothers supplied information on doctor-diagnosed childhood infections up to four years of age. For both sexes of children combined, the adjusted OR for total infectious diseases for the fourth quartile versus the first quartile of maternal serum PFOS was elevated and statistically significant (OR = 1.61, 95% CI: 1.18, 2.21). The trend across quartiles was statistically significant (p = 0.008). The fourth quartile versus first quartile OR was also statistically significant for boys (OR = 1.59, 95% CI: 1.03, 2.46) and girls (OR = 1.71, 95% CI: 1.08, 2.72) separately, although the trend was statistically significant only for girls (p = 0.036). Detailed data on associations between maternal serum PFOS and individual infections were not provided.

2.5.3.5. Granum et al. (2013). This prospective birth cohort study in Norway (n = 65–93, mean maternal serum PFOS = 5.6 ng/ml) found no association between parentally reported incidence of colds, gastroenteritis, or otitis media at 3 years of age. It is notable that the Granum et al. (2013) study had a small sample size compared to any of the positive studies (Impinen et al., 2018; Dalsager et al., 2016; Goudarzi et al., 2017; and Fei et al., 2010).

2.5.3.6. Okada et al. (2012). In this prospective cohort study in Sapporo, Japan (n = 343, mean maternal serum PFOS = 5.6 ng/ml), questionnaire data were collected for maternally identified infectious diseases at 18 months of age. Due to the overall low incidence of reported infectious diseases, the authors only reported on otitis media, which was not significantly associated with maternal serum PFOS.

2.5.3.7. Looker et al. (2014). This study was conducted in an adult population based in the C8 Science Panel in Ohio and West Virginia (U.S.) (n = 755, geometric mean serum PFOS = 8.32 ng/ml). The study found no significant associations between self-reported occurrence of cough, colds, flu, or other respiratory infections for the previous 12 months and concurrent PFOS exposure.

2.6. Considerations for the interpretation of infectious disease studies

These studies addressed different age ranges, including different age ranges among children. They also evaluated somewhat different endpoints of infectious disease and provided somewhat different statistical measures of association. Nonetheless, four of the seven studies found statistically significant associations between levels of population exposure to PFOS and the incidence of infectious disease. This includes four of the six studies of infectious disease in children. It is particularly notable that the serum PFOS concentrations in these populations closely mirrored PFOS serum concentrations in the general U.S. population (CDC, 2017). Overall these studies provide evidence for an association between general population levels of PFOS exposure and infectious disease, a clinical meaningful measure of health risk.

2.7. Overall conclusion regarding human epidemiological data on immunosupression

In summary, the cohort studies provide evidence that PFOS is

Table 3
Summary of epidemiological studies assessing the association between PFOS exposure and infectious disease.

Study	Ages addressed by outcome ascertainment (n)	Serum PFOS concentration	Source of outcome assessment data	Statistically significant association with PFOS exposure	Measure/strength of association
Impinen et al. (2018) (prospective birth cohort)	0–10 yrs old (641)	Mean = 5.6 ng/ml (cord serum)	Parental interview at 10 yrs old	Lower respiratory tract infections from 0–10 yrs old	**β (95% CI)** 0.50 (0.42, 0.57)[a]
Dalsager et al. (2016) (longitudinal prospective cohort)	1–4 yrs old (346)	Median = 8.07 ng/ml (maternal serum, < gestational-week 16)	Maternal reporting every two weeks	Number of days with fever	**Adj. IRR (95% CI)** Highest vs lowest 1.65 (1.24, 2.18)[a]
Fei et al. (2010) (prospective birth cohort)	6–11 yrs old (577)[b]	Mean = 5.6 ng/ml (maternal serum, gestational week 4–14)	Danish National Hospital Registry	Hospitalization for infection	**Adj. IRR (95% CI)** *Girls only* 3rd vs. 1st quartile 1.61 (1.05, 2.47) 4th vs. 1st quartile 1.59 (1.02, 2.49)
Goudarzi et al. (2017) (prospective birth cohort)	< 4 yrs old (1,075)	Mean = 5.5 ng/ml (maternal serum, third trimester)	Maternal reporting of physician diagnoses at 4 years old	Total infectious diseases	**Adj. OR (95% CI)** *All children* 4th vs. 1st quartile 1.61 (1.18, 2.21) sig. for trend *Boys* 4th vs. 1st quartile 1.59 (1.03, 2.46) not sig. for trend *Girls* 4th vs. 1st quartile 1.71 (1.08, 2.72) sig. for trend
Granum et al. (2013) (prospective birth cohort)	0–3 yrs old (65–93)[c]	Mean = 5.6 ng/ml (maternal plasma at delivery)	Maternal reporting at 1, 2 and 3 years old	X[d]	—
Okada et al. (2012) (prospective birth cohort)	0–18 months (343)	Mean = 5.6 ng/ml (maternal serum post-second trimester or at delivery)[e]	Maternal reporting of physician diagnosis at 18 months[f]	X	—
Looker et al. (2014) (prospective cohort)	Adults, previous 12 months[g] (755)	Geometric mean = 8.3 ng/ml (at time of outcome ascertainment)	Participant interview	X	—

(Note: In each study details are reported only for those associations that were statistically significantly positive for childhood infections. The reader is referred to the original studies for full details.)
β = adjusted Poisson regression estimate; Adj. = adjusted; CI = confidence interval; IRR = incidence rate ratio; OR = odds ratio

[a] based on Bonferroni correction

[b] 1,400 children were followed, of which, 363 were hospitalized for infection. Since some had multiple hospitalizations, the total number of hospitalizations was 577. The analysis was carried out on the number of hospitalizations

[c] considers all analyses: common cold, gastroenteritis; quantal and dichotomous

[d] No statistically significant finding

[e] Maternal blood samples obtained following second trimester except in cases of anemia. The authors do not specify the number of samples taken at each time point

[f] Data were collected on otitis media, febrile convulsions, respiratory syncytial virus (RSV), chicken pox, bronchitis, rhinitis, pneumonia, skin infection, other viral infection. However, due to low incidence, analysis was conducted on otitis media only

[g] Authors do not provide summary statistic for population age. Median age ≈ 41–50 yrs

associated with a decrease in some vaccine antibody responses following vaccination. Further, we conclude that there is evidence of an increase in childhood infections that is associated with gestational PFOS exposure. A decrease in vaccine antibodies and an increase in childhood infections are mutually consistent, since both are indicative of immunosuppression. Therefore, the epidemiologic evidence provides support for the human relevance of an RfD based on immunosuppression from animal data. The strength of this support is tempered somewhat by the inability to quantify an independent effect of PFOS in human studies relative to PFOA and/or other co-occurring PFAS. Nonetheless, the evidence from human studies and the PFOS-specific evidence from the animal studies are mutually reinforcing, and the specificity of the animal evidence points toward an independent effect of PFOS in the human data. In addition, the NTP (2016) systematic review of the immunotoxicity of PFOS and PFOA concludes that there is a "moderate" level of evidence from human studies that PFOS is an immune hazard.

2.8. Overall conclusion from Hazard Identification

We evaluated toxicological studies from experimental animals, rather than human epidemiological studies, for use as the quantitative basis of the PFOS RfD. While the epidemiology data are more directly relevant to the conditions and extent of human exposure, they are not suited for derivation of an RfD for several reasons. First, the nature of the epidemiology studies does not lend them to the identification of a point-of-departure (e.g., a NOAEL) as the association between serum PFOS concentration and vaccine antibody levels was not stratified by serum PFOS concentration in many of these studies. Second, as discussed above, the highly correlated co-occurrence of other PFAS prevents determination of PFOS-specific effects.

Based on the information presented above, we identify decreased PFC response as a highly sensitive adverse endpoint for PFOS exposure that is supported by the human data on vaccine response and childhood infectious diseases. Our analysis of the available immunotoxicity studies of PFC response, has identified Dong et al. (2009) as the most appropriate study for the derivation of an RfD for PFOS.

3. Exposure-Response Analysis for decreased PFC response from Dong et al. (2009)

The ultimate goal of this assessment was to derive a chronic human intake dose (i.e., an RfD, ng/kg/day) for PFOS. However, given the much longer half-life of PFOS in humans as compared to experimental animals, interspecies comparison of exposures on the basis of intake dose is problematic. This is because a given intake dose of PFOS results in a much higher serum PFOS level in humans than in experimental animals (e.g. mice). Therefore, internal exposure, as measured by serum PFOS concentration, was used as the exposure metric in the exposure-response analysis. This approach also has the advantage of normalizing any interspecies differences in absorption, distribution, and elimination (Note that PFOS is not metabolized).

As illustrated in Fig. 2, the exposure-response approach starts with a point-of-departure (POD; e.g. NOAEL, LOAEL, or BMDL) based on an animal serum PFOS concentration. Five standard uncertainty factors are considered for application to the POD to derive a Target Human Serum Level that is analogous to the RfD, but expressed in terms of serum concentration rather than administered dose. The corresponding RfD is then derived by application of a PFOS-specific clearance factor (L/kg/day) that relates human serum concentration (ng/L) to intake dose (ng/kg/day).

3.1. Exposure-response modeling

When possible, the POD is derived from the exposure-response data through benchmark dose modeling (USEPA, 2012). We attempted

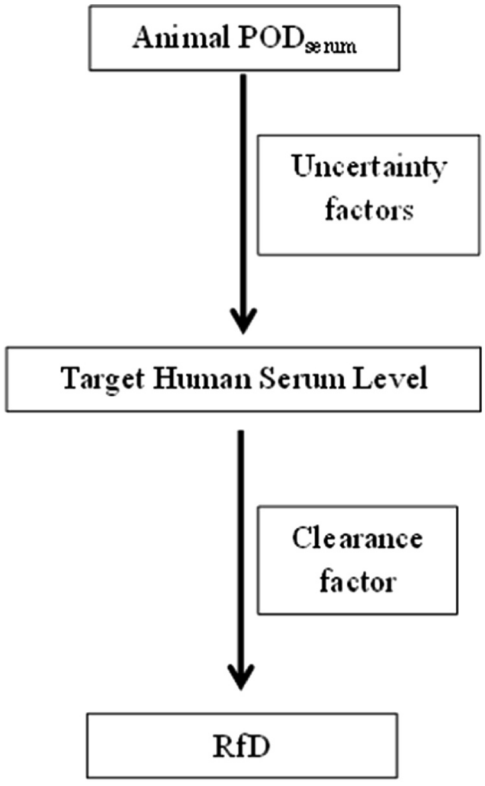

Fig. 2. Schematic of the PFOS RfD derivation process based on serum PFOS concentration.

benchmark dose modeling of the data for the decreased PFC response endpoint from the Dong et al. (2009) study using USEPA benchmark dose software (BMD software, ver. 2.6.0.1). Using all six data points for PFC response from Dong et al. (2009), none of the available benchmark dose models gave an acceptable fit. This was due, in part, to a disproportionally large decrease in PFC response at the highest dose that was possibly indicative of a stress response (e.g., increased serum corticosterone) and/or splenic cytotoxicity (data not shown). Therefore, benchmark dose modeling was attempted with the omission of the highest dose. Although several models gave ostensibly acceptable fits to these data, the BMDS software identified that these data did not meet the criteria for an assumption of constant variance. In addition, the software was unable to calculate a BMDL under the assumption of non-constant variance. This was likely due to the steepness of the dose-response in the vicinity of the BMD (DWQI, 2018).

When no BMDL can be derived, a NOAEL or LOAEL is used as the POD (USEPA, 2012). We identified the NOAEL serum concentration of 674 ng/ml for decreased PFC response from Dong et al. (2009) as the POD.

3.2. Application of uncertainty factors

We considered the application of the following five standard uncertainty factors (UFs) (USEPA, 2002):

UF$_{sub\text{-}chronic}$ = 1: Applied to the sub-chronic animal POD$_{serum}$ to estimate the corresponding NOAEL for a chronic duration study. Dong et al. (2009) was a 60-day study and can, therefore, be considered to be of sub-chronic duration. No chronic exposure (≥ 90 days) studies of PFC response were identified that can provide direct evidence as to whether decreases in PFC response observed with sub-chronic duration of exposure would progress (i.e., exhibit a greater magnitude of response) with chronic exposure. However, the available studies of PFC response with PFOS administration cover a range of exposure from 7 to 60 days (see Table 1). Fig. 3 presents a comparison from these studies of

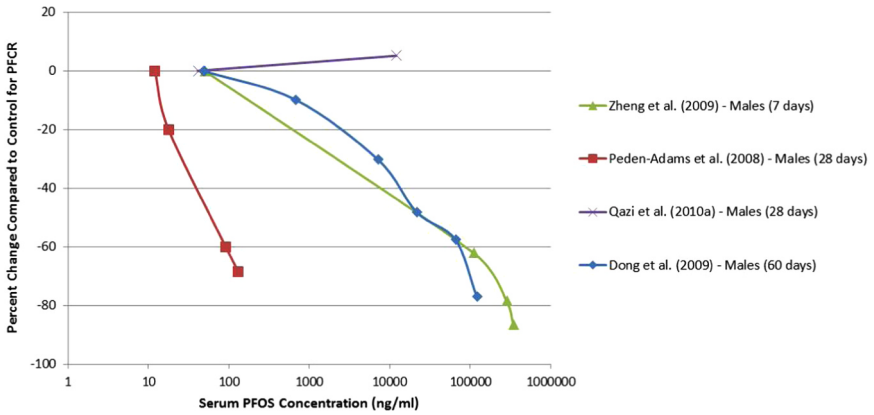

Fig. 3. Comparison of percentage change from controls for PFC response (PFCR) studies that reported serum PFOS concentrations.

the percent decrease in PFC response from the control value as a function of serum PFOS concentration and the duration of dosing.

Note that Zheng et al. (2009) (7-day duration) and Dong et al. (2009) (60-day duration) both achieved a 60% decrease in PFC response compared to their respective controls at a serum PFOS concentration of approximately 100,000 ng/ml. Furthermore, over the range of exposure duration from 7 to 60 days (including the 28-day Peden-Adams et al. (2008) study), a 60% reduction in PFC response occurs over a three-order of magnitude range of doses. This strongly suggests that the decrease in PFC response does not progress with longer exposure to a given serum PFOS concentration over this range of exposure durations, and suggests that in the absence of direct toxicity to the spleen, the decrease in PFC response would not progress with chronic exposure.

$\mathbf{UF_{LOAEL}}$ = 1: Applied to the animal POD_{serum} based on a LOAEL to estimate the corresponding NOAEL, when no NOAEL is identified in the study under consideration. For decreased PFC response as reported in Dong et al. (2009), the POD is derived directly from the NOAEL.

$\mathbf{UF_{animal}}$ = 3: Applied to the animal POD_{serum} to address differences between average laboratory animals and average humans in both toxicokinetics and toxicodynamics. A factor of 3 (i.e., one-half log unit) is normally assigned to each. In this case, the animal POD_{serum} is based on serum PFOS concentration and, as described above, this approach addresses interspecies toxicokinetic differences. Therefore, a value of 3 is applied to account for potential toxicodynamic differences between mice and humans.

$\mathbf{UF_{human}}$ = 10: Applied to account for the range of sensitivity for a given chemical and a given adverse effect between an average human and sensitive elements of the population. As is the case for PFOS, in the absence of specific evidence of a smaller range of sensitivity, the full UF of 10 is the standard default assumption.

$\mathbf{UF_{database}}$ = 1: Applied if there is concern that future studies may identify a more sensitive effect, target organ, population, or lifestage. As detailed elsewhere (DWQI, 2018), the database for PFOS contains adequate studies addressing reproductive, developmental, and neurological/neurobehavioral effects as well as systemic adult effects.

The combination of UFs is treated multiplicatively (USEPA, 2002). The individual UFs shown above give a total UF of 30. Dividing the POD (based on animal serum PFOS concentration) of 674 ng/ml gives a Target Human Serum Level of 22.5 ng/ml.

3.3. Reference dose derivation

To derive an intake dose as an RfD (ng/kg/day) corresponding to the Target Human Serum Level (ng/L) requires a toxicokinetic-based factor that relates these two dose metrics. This is referred to as a clearance factor (CL; L/kg/day). An estimate of the PFOS-specific CL was derived by the USEPA (2016b) from estimates of the PFOS-specific volume of distribution (Vd; L/kg) and the PFOS half-life ($t_{1/2}$; days). The relationship among these variables is given as:

$$CL = Vd \times (\ln 2 / t_{1/2})$$

The relationship among the PFOS intake dose and the serum PFOS concentration at steady state is then defined in the following equation:

$$RfD(ng/kg/day) = \text{Target Human Serum Level } (ng/L) \times CL\ (L/kg/day)$$

The mean half-life of 5.4 years from Olsen et al. (2007) was used in the derivation of the USEPA CL for PFOS. This half-life estimate was based on a study of retired workers (n = 26; age 55–75 years at baseline), all but two of whom were men, who had significant occupational exposure (median baseline serum concentration = 626 ng/ml) to PFOS at the start of the study. The large body burden of the subjects in this study, compared to the general population, minimized the effects of background PFOS exposures. The arithmetic mean half-life estimated from this group was 5.4 years (1976 days) and the median was 4.6 years (1661 days). However, the range was 2.4–21.7 years, indicating significant inter-individual variability. Although the large body burden resulting from extended occupational exposure raises the possibility of saturation kinetics of elimination, semi-log plots of serum concentration versus time for each subject show no evidence of significant deviations from the linear relationship expected under first-order elimination kinetics (Olsen et al., 2007).

A study by Li et al. (2018) estimated the half-life of PFOS based on decline of serum PFOS levels after exposure to contaminated drinking water ended. The sample used to derive the estimate of PFOS half-life was 106 subjects, ranging from 4 to 83 years old at baseline, of which 20 were men and 30 were women 15–50 years old. The estimates of half-life for the full sample as well as for men and women 15–50 years old are presented separately by the authors. The median serum PFOS concentration at the initial collection was 345 ng/ml (55% of the median in Olsen et al., 2007). The mean half-life estimates were 3.4 years for the entire study population, 3.1 years for women, and 4.6 years for men. The half-lives for the men (95% CI = 3.7–6.1 years) had greater inter-individual variability than for the women (95% CI = 2.7–3.7 years), and the half-lives of some subjects were very long (8–10 years with one value greater than 10 years). While data for those younger than 15 years were not reported separately, it appears that the half-life in men was also longer than for children. The estimated mean half-life for men age 15–50 of 4.6 years is in reasonable agreement with the estimate from Olsen et al. (2007), despite the larger starting body burden in the Olsen et al. (2007) study group. However, given that the men in Olsen et al. (2007) were all older than the men age 15–50 in Li et al. (2018), the studies may not be directly comparable. Nonetheless, the results from Li et al. (2018) appear to support the half-life estimate from Olsen et al. (2007) that was used for derivation of the CL by USEPA (2016b).

The CL derived by USEPA (2016b) using Olsen et al. (2007) mean estimate of half-life (5.4 years) and an estimate of the PFOS volume of distribution of 0.23 L/kg, is 8.1×10^{-5} L/kg/day. Multiplying this CL by the Target Human Serum Level of 22.5 ng/ml (2.3×10^{4} ng/L) gives an intake dose (i.e., the RfD) of 1.8 ng/kg/day.

4. Discussion

Although this assessment focuses on the immunotoxicity of PFOS, this focus is underlain by our much broader effort that served as the basis of a comprehensive health effects assessment of PFOS. This effort is documented elsewhere (DWQI, 2018). As part of this effort, a search and assessment of the PFOS animal toxicity and epidemiology literature identified multiple endpoints from PFOS exposure (e.g., developmental, endocrine, hepatic, immune, cancer) with decreased PFC response in mice emerging as the most sensitive and appropriate endpoint for derivation of an RfD for PFOS.

4.1. Scientific issues

An independent assessment of PFOS (DWQI, 2018), as well as other efforts discussed below, identified immunosuppression as an appropriate basis for the derivation of a PFOS RfD. Several scientific issues relevant to the decreased PFC response endpoint are discussed below.

4.1.1. Species of test animal

Each of the five available studies of the effect of PFOS exposure on the PFC response endpoint was conducted in mice, the species in which this effect is typically evaluated (Kaminski et al., 2008). No PFC response data for PFOS was identified in other species. Therefore, the relative sensitivity of mice compared to other common test species or to humans for this endpoint with respect to PFOS exposure is uncertain.

4.1.2. Strain and sex

The five PFC response studies used two strains of mice, either C57BL/6 (Dong et al., 2009; Zheng et al., 2009) or B6C3F1 (Keil et al., 2008; Peden-Adams et al., 2008; Qazi et al., 2010). The relative sensitivities of these strains to PFOS effects on the PFC response is uncertain, although both Peden-Adams et al. (2008), showing the most sensitive response, and Qazi et al. (2010), showing no response, used the same strain. Of the available PFC response studies in adult mice, the only study using both sexes (Peden-Adams et al., 2008) found that males were more sensitive than females. However, as this is a single observation, no definitive conclusions can be made about relative sensitivity of males versus females. We note that in the developmental immunotoxicity study of Keil et al. (2008), male pups were also more sensitive than female pups. However, it is unclear whether this finding can be generalized to adult animals.

4.1.3. Gestational versus adult exposure

The potential for gestational exposure to PFOS to cause immunotoxicity, and specifically decreased PFC response, is of interest because PFOS is well-established as a developmental toxicant (as reviewed in DWQI, 2018). With the exception of the Keil et al. (2008) study, all of the mouse studies examined PFC response following exposure in adult animals. Because Keil et al. (2008) did not provide serum PFOS concentrations, the sensitivity of the PFC response to gestational exposure in that study cannot readily be compared to the response to adult exposure. It is, therefore, uncertain whether gestational and lactational exposures resulting from dosing of dams with PFOS results in a more sensitive PFC response.

4.1.4. Route of exposure

All of the PFC response studies except the dietary study by Qazi et al. (2010) administered PFOS by gavage, and Qazi et al. (2010) was the only study that did not show a decrease for PFC response. While it could be hypothesized that the negative results in Qazi et al. (2010) can be explained by the dietary exposure route, this does not seem likely. Potential differences in absorption of PFOS due to dosing route are accounted for by the use of internal dose (serum PFOS level) as the dose metric. Additionally, for some contaminants with short half-lives, greater toxicity may occur from the higher peak serum levels that result from bolus (e.g. gavage) dosing than from more continuous (e.g. dietary) dosing. However, for a daily bolus dose of a chemical with a long half-life, such as PFOS (40 days in male mice; Chang et al., 2012), the critical factor determining short term concentration at the target organ is the number of doses per half-life. In the case of Dong et al. (2009) where mice were dosed by gavage once each day, there were 40 doses during each PFOS half-life. From a basic toxicokinetic standpoint, this rate of dosing would result in insignificant short-term fluctuations in serum and target organ PFOS concentration. The average concentration over the course of the dosing period (i.e, the area under the curve) will be essentially equivalent for gavage and dietary exposure over the 60-day dosing period in Dong et al. (2009).

Furthermore, if the same administered PFOS dose given by gavage administration resulted in a larger transient serum PFOS concentration compared to dietary administration, the ratio of administered dose to the short-term serum PFOS concentration would be expected to be lower for gavage administration. A comparison across these studies of the ratio of administered dose to serum PFOS concentration at the LOAEL dose or NOAEL dose (Qazi et al., 2010) approximately 24 h after the last administered PFOS dose indicates that ratio for Qazi et al. (2010) is comparable to the Dong et al. (2009) and Peden-Adams et al. (2008) ratios, but less than the ratio for Zheng et al. (2009) (see Supplemental Information, Table S1, for details). This suggests that, averaged over 24 h, the kinetics of absorption and distribution were not dependent on the route of exposure.

For all of the above reasons, there does not appear to be a significant issue in the derivation of an RfD based on a study employing gavage exposure.

4.1.5. PFOS in the serum of control animals

Given the ubiquity of PFOS in the environment, it is not surprising that low levels of PFOS (48 ng/ml, 7% of the level in the lowest dosed group, in Dong et al., 2009) were found in the sera of control animals (e.g., likely through the contamination of rodent chow or from laboratory contamination). At least in Dong et al. (2009) and Peden-Adams et al. (2008), the presence of PFOS in the control animals could potentially have resulted in overestimating the NOAEL and/or LOAEL (depending on the specific study), resulting in a larger RfD (see Table 4).

4.1.6. PFC response of control animals

The PFC response in the control animals in Dong et al. (2009) (597 per 10^6 splenocytes) is lower than the response in any of the four remaining studies (range 2300 to 7800 per 10^6 splenocytes) (see Table 1). The reasons for this may include inter-individual differences in SRBC antigenicity among donor sheep, differences among commercial suppliers of mice, animal husbandry, and diets, as well as intra-strain genetic drift. The differences in mouse strains among the studies is not likely to be the explanation for the decreased PFC response in control mice in Dong et al. (2009) since Zheng et al. (2009) achieved a PFC response in control mice approximately six times that of Dong et al. (2009) using the same strain of mouse.

As discussed above, the serum PFOS concentration in the control mice in Dong et al. (2009) (48 ng/ml) was relatively high compared to that in Peden-Adams et al. (2008) (12 ng/ml). However, this is also not likely to explain the low control PFC response in Dong et al. (2009). The serum PFOS concentration in the control animals in Qazi et al. (2010) was only slightly smaller (41 ng/ml) than the concentration in Dong et al. (2009) control mice, but the control PFC response in Qazi et al. (2010) was the highest among the five studies and about 13 times that

Table 4
Serum PFOS concentrations in control animals relative to the serum PFOS concentration at the NOAELs from PFC response studies.

Study	Serum PFOS concentration in control animals (ng/ml)	Serum PFOS concentration in control animals as a percent of serum PFOS concentration at the NOAEL for PFC response
Dong et al. (2009)	48	7%
Peden-Adams et al. (2008)	12 (M)	68% (M)
	17 (F)	14% (F)
Zheng et al. (2009)	< 50[a]	—[b]
Qazi et al. (2010)	41	0.3%

[a] Not detected. The value in the table is based on the detection limit.
[b] No NOAEL was identified.

in the Dong et al. control mice. Although the reason for the lower PFC response among control animals in Dong et al. (2009) is not clear, it suggests the possibility that the PFC response of the Dong et al. (2009) mice may have been attenuated at all doses, resulting in overestimating the true serum PFOS LOAEL from that study.

It is notable that each of the scientific issues discussed above suggests the potential for the derivation of a smaller PFC response-based RfD than the one derived here from Dong et al. (2009). We have not identified significant uncertainties in the use of Dong et al. (2009) that suggest the potential for a larger RfD.

4.2. Support for the identification of immunotoxicity as the critical effect

As discussed below, the conclusion of this assessment regarding the use of immunotoxicity as the critical effect for an RfD for PFOS agrees with previous assessments and review articles that identify immunotoxicity as a credible effect of PFOS and/or advocate its use for quantitative risk assessment.

4.2.1. Human health implications of decreased PFC response

Decreased PFC response in mice due to a decreased IgM response to a foreign antigen is a well-recognized indicator of immune function that has previously been used in the development of RfDs by USEPA under its Integrated Risk Information System program (USEPA, 2010, 2011). This endpoint is analogous to decreased antibody response in humans and can indicate an increased disease risk. PFC response is “a well-accepted measure of immune function included in many guidelines or testing requirements for immunotoxicity” (NTP, 2016). As a predictor of overall immune function, assessment of antigen-specific antibodies (e.g., IgM) reflects the interaction of various immune cell types (e.g., T- and B-cells, antigen presenting cells) to mount a response to an immune challenge (e.g., T-cell-dependent antigen) (NTP, 2016). Therefore, the consistent observation of decreased PFC response in mice and the decrease in antibody response observed for different vaccines from human studies, as discussed above, support the use of immunosuppression, specifically, PFC response in mice as the basis for a PFOS RfD. This is further supported by the association of the incidence of childhood infections with PFOS exposure.

4.2.2. Support from other sources for the identification of immunosuppression as a valid endpoint

An in-depth examination of PFOS immunotoxicity by the National Toxicology Program supports the identification of immunosuppression as a well-established effect of PFOS exposure (NTP, 2016). NTP conducted a systematic review of immunotoxicity of both PFOS and PFOA based on epidemiological, animal, and mechanistic studies. The NTP concluded that there was “moderate confidence that exposure to PFOS is associated with suppression of the antibody response based on the available human studies” and “high confidence that exposure to PFOS is associated with suppression of the antibody response based on the available animal studies.” NTP's overall conclusion was that PFOS is “presumed to be an immune hazard to humans”. In considering the mechanisms underlying the suppression of antibody response, NTP noted that such mechanisms were “not well understood” and were, as suggested by animal studies, PPARα-independent.

As part of the NTP systematic review, risk of bias analyses were conducted for individual studies that informed hazard identification conclusions. This analysis was conducted to assess whether the relationship between exposure and outcome was affected by study design and conduct (NTP, 2015, 2016). For Dong et al. (2009), NTP assigned “probably high risk of bias” ratings due to lack of researcher blinding in allocation concealment, dose administration, and outcome assessment. In each of these categories, these elements were not reported in the study (as is the case for most toxicity studies reported in peer-reviewed journals and other formats) and this information could not be ascertained when NTP contacted the study authors. However, ratings of “definitely low risk of bias” and “probably low risk of bias” were given to all of the other elements (i.e., randomization of animals to dose groups, identical experimental conditions across dose groups, complete data reporting without attrition or exclusion, confidence in exposure characterization, complete data reporting, and appropriate statistical analyses) assessed for this study.

USEPA (2016a, 2016b) identified PFC response as reported by Dong et al. (2009) and Peden-Adams et al. (2008) as among the most sensitive effects, based on administered dose following PFOS exposure in animals. The USEPA (2016b) further stated that decreased antibody titers in humans and decreased PFC response in animals “indicates a concern for adverse effects on the immune system.” However, their assessment did not use immunotoxicity as an endpoint for deriving an RfD for PFOS due to “lack of human dosing information and lack of low-dose confirmation of effects in animals for the short-duration study.” We find this rationale to be incompletely described and poorly justified.

Subsequent to the release of the USEPA (2016a, 2016b) documents on PFOS, review articles by Dong et al. (2017) [note: different author than Dong et al. (2009)] and Lilienthal et al. (2017), as well as risk assessments developed by the Minnesota Department of Health (MDH, 2017) and the Agency for Toxic Substances and Disease Registry (ATSDR, 2018) have noted that immunotoxicity is an important effect of PFOS. Dong et al. (2017) and Lilienthal et al. (2017) pointed out the greater sensitivity of some of the immunotoxic endpoints of PFOS, including decreased PFC response, compared to the developmental effect that served as the basis for the USEPA RfD. Lilienthal et al. (2017) concluded that the immune system effects from PFOS exposure “likely constitute a sound basis for ongoing and future regulations.” Furthermore, both the MDH (2017) Reference Dose and the ATSDR (2018) Minimum Risk Level (MRL) incorporate modifying factors (3 and 10, respectively) to account for immunotoxicity as a more sensitive toxicological endpoint than reduced rat pup weight in Luebker et al. (2005), the critical effect for the USEPA (2016a) Health Advisory. ATSDR (2018) concluded that immunotoxicity is a valid and relevant basis for risk assessment but did not use it as the critical effect due to the lack of a pharmacokinetic model to estimate the time weighted average serum PFOS concentrations in the relevant mouse strains. It is notable that the ATSDR (2018) MRL of 2 ng/kg/day, based on application of a modifying factor for more sensitive immunotoxic effects, is essentially identical to the RfD presented herein of 1.8 ng/kg/day,

based on immunotoxicity as the critical effect.

4.3. Risk characterization

The RfD of 1.8 ng/kg/day derived from the Dong et al. (2009) study is based on a PFOS Target Human Serum Level of 22.5 ng/ml. This concentration was derived from the mouse NOAEL in Dong et al. (2009) and modified by uncertainty factors to account primarily for toxicodynamic differences between mice and humans and for the range of potential sensitivities in the human population. As such, the RfD and the Target Human Serum Level are intended to represent lifetime exposure levels that will be without significant adverse effect, including to sensitive human subpopulations. One approach for investigating whether these exposure levels can provide the intended level of protection is to compare the PFOS Target Human Serum Level to the PFOS serum concentrations identified in the epidemiologic literature as being associated with statistically significant decreases in vaccine response.

As documented in Table 2, the central tendency estimates of serum PFOS concentrations from the studies that found associations with decreased vaccine response in one or more vaccines range from 5.6 to 27.3 ng/ml. The Target Human Serum Level derived here (22.5 ng/ml) falls within the upper end of this range. However, this comparison should be considered in the light of several important caveats.

- The epidemiology studies cannot necessarily be used to describe a dose-response continuum across studies due to issues of comparability in timing of response ascertainment, age at serum measurement (fetal, childhood, adult), and vaccine type.
- The nature of these studies only permits the determination of a statistical association between the overall exposure in a population (characterized as a central tendency estimate of serum PFOS concentration) and the overall extent of vaccine response. Therefore, it cannot be determined from these data whether the observed association is driven primarily by the highest exposed individuals or by the full range of exposures in the study population.
- Finally, as discussed in the Epidemiology section, although the associations noted in Table 2 were derived from analysis of the reported serum PFOS concentrations, none of these studies reported analyses for PFOS that controlled for the serum concentrations of other PFAS. Therefore, it is not known to what extent the reported associations reflect the unique contribution of PFOS to the decreased vaccine response.

Given these caveats, caution should be exercised in assessing the public health protectiveness of the Target Human Serum Level and associated RfD on the basis of the available epidemiology data on immunotoxicity. However, while the caveats mentioned above preclude a definitive conclusion as to whether the Target Human Serum Level and RfD are sufficiently low, comparison to the epidemiology data strongly suggests that the RfD is not overly conservative. Ultimately, the RfD derived from Dong et al. (2009), which incorporates appropriate uncertainty factor adjustments addressing the extrapolation from animal data to human health risk, is intended to be public health protective and to appropriately minimize PFOS exposure based on available evidence. Nonetheless, we acknowledge the inherent uncertainty in comparing the predictions of the RfD based on animal data and the epidemiology data regarding immunotoxicity. Additional scientific research may resolve this uncertainty.

The 2013–2014 NHANES data (CDC, 2017) provide summary serum PFOS data for a representative sample of the U.S. population 12-years old and older. The geometric mean and median total serum PFOS concentrations are 4.99 and 5.20 ng/ml, respectively. The 95th percentile is 18.5 ng/ml. Ye et al. (2017) provides parallel data from the same NHANES database for children 3–11 years old. The geometric mean and median total PFOS concentrations are 3.88 and 3.75 ng/ml, respectively, and the 95th percentile is 11.0 ng/ml. A comparison of the PFOS Target Human Serum Level of 22.5 ng/ml to these data suggest that less than 5% of the U.S. population 12-years old and older (i.e., the population represented in the NHANES database) has a serum PFOS concentration that exceeds 22.5 ng/ml, while few if any children under 12 years old in the U.S. have concentrations that exceed this level (Ye et al., 2017).

The USEPA's Office of Water (USEPA, 2016a, 2016b) has published risk-based drinking water guidance for PFOS. This guidance derived an RfD of 2×10^{-5} mg/kg/day (20 ng/kg/day) based on reduced rat pup weight in a two-generation study (Luebker et al., 2005). Based on the CL of 8.1×10^{-5} L/kg/day, the USEPA RfD corresponds to a human serum concentration of 247 ng/ml. The USEPA (2016a, 2016b) RfD and corresponding serum concentration are an order of magnitude larger than the RfD and its corresponding Target Human Serum Level based on the immune endpoint of decreased PFC response from Dong et al. (2009) derived here. With respect to central tendency estimates of serum PFOS concentrations that have been associated with reduced vaccine response in epidemiology studies (i.e., a range from 5.6 to 27.3 ng/ml), the serum PFOS concentration corresponding to the USEPA RfD is approximately an order of magnitude larger than the upper end of the range. As such, the USEPA RfD may not be protective of decreases in vaccine response in humans, and ongoing PFOS exposure at this level may have the potential to reduce the protectiveness of vaccines at the clinical level.

5. Overall summary and conclusion

- We conclude that decreased PFC response in mice is a valid indicator of immunosuppression and is an adverse effect that is relevant to the human health risk from PFOS exposure.
- This conclusion is consistent with and supported by epidemiologic evidence for immunosuppression. Epidemiology studies identify associations of decreased vaccine response and increased risk of childhood infections for estimates of PFOS exposure that are consistent with PFOS exposures in the U.S. general population.
- Of the identified toxicological studies assessing PFC response and PFOS exposure, we conclude that the Dong et al. (2009) study in mice is a scientifically valid and appropriate basis for the derivation of an RfD.
- Based on exposure-response analysis of serum PFOS concentrations and PFC response from the Dong et al. (2009) study, along with the application of uncertainty factors and use of a clearance factor for conversion to an intake dose, an RfD of 1.8×10^{-6} mg/kg/day was calculated.
- This RfD is intended to be public health protective. It is not excessively restrictive, as the corresponding serum PFOS concentration is comparable to population levels of exposure to PFOS that have been associated with decreased vaccine response and increased childhood infections in epidemiology studies. It is noted that the corresponding serum PFOS concentration is well above levels of exposure that are currently prevalent in the U.S. general population. Nonetheless, this RfD appropriately minimizes PFOS exposure based on available evidence.
- A weight of evidence/evidence integration evaluation, based on the Hill criteria, was conducted to determine the relevance of the PFC response data from animals to human health (See Appendix B). Based on PFC response data in animals and decreased vaccine response in humans, it was concluded that suppression of antibody response is a relevant human health endpoint for environmental exposure to PFOS, and the weight of evidence was judged to be medium-high.

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Authors' contributions

BP and AS conceived the structure of the manuscript. AS and BP evaluated and analyzed the data discussed in the assessment and wrote the manuscript. GP provided substantive technical input to the manuscript. All authors provided critical review of the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest of any sort relating to the subject matter of this manuscript.

Appendix A

Other endpoints identified in the Dong et al. (2009) study

Dong et al. (2009) also reported results for other functional or observational immune endpoints. Natural killer (NK) cell activity increased with dose, reaching statistical significance in the 0.08 mg/kg/day group and declined at higher doses. This decline reached statistical significance (relative to controls) at 0.83 mg/kg/day. While this increase was observed at the same dose as the LOAEL for decreased PFC response, the biological significance of this NK cell activity observation is uncertain given its non-monotonic response. Decreased relative spleen and thymus weights and decreased splenic and thymic cellularity were observed at doses $\geq$ 0.42 mg/kg/day. Additionally, decreases in lymphocyte subpopulations and lymphocyte proliferation were generally observed at doses $>$ 0.42 mg/kg/day. Observations regarding measurement of serum corticosterone in this study are addressed in the main text.

Increased relative liver weight in Dong et al. (2009) was observed with a NOAEL of 0.008 mg/kg/day (674 ng/ml). The NOAEL for this endpoint is the same as that for the PFC response endpoint. However, as explained in the DWQI (2018) assessment, increased relative liver weight from Dong et al. (2009) was not used as the basis for an RfD based on dose-response modeling considerations (DWQI, 2018). Other toxicologically relevant endpoints (e.g., decreases in body weight, food intake, relative kidney weight) occurred at doses of 0.42 mg/kg/day or greater.

Appendix B

Evidence integration

The main part of this paper describes a traditional risk assessment approach, employing hazard identification, dose-response assessment and risk characterization, that results in the conclusion that PFOS-mediated suppression of antibody response is a relevant human health endpoint for environmental exposure to PFOS. In addition, a quantitative expression of that conclusion is provided in the form of an RfD.

In this Appendix, we present a separate, independent evaluation of the strength of that conclusion against a well-recognized set of evaluative criteria, the Hill (1965) criteria for causality. We evaluate the epidemiology and animal data for antibody response and plaque forming cell response, respectively, using applicable Hill criteria. This evaluation is further guided by quoted and italicized elaborations below each Hill criterion for evaluation of generic epidemiology data by the USEPA's Integrated Risk Information System program (2013). These elaborations (although not specifically applied to the animal data by the USEPA) can generally be applied to the interpretation of the animal data as well. We separately evaluate the epidemiology and animal evidence streams and then integrate the two streams to produce an overall evaluation of the weight of evidence. The Hill Criteria are intended to provide a descriptive basis upon which to assess the evidence for causality. As such, the weighing of the strength of evidence for each individual criterion and for the criteria as a whole, necessarily depends on professional judgement. We first provide a narrative description of our assessment of each criterion. Based on this description, we then assign a score on the continuum of high to low. Based on the individual scores, as well as on our judgement regarding the relative weight of each of the criteria, we then provide our overall assessments for the epidemiology and animal evidence, and for the integrated epidemiology and animal evidence on the continuum from high to low. We recognize that the use of professional judgement in this ranking procedure, while informed, is nonetheless subjective. Others may reach different conclusions regarding the appropriate descriptors of causality.

Epidemiology data are included in this evidence integration because such data were available and provide useful information that strengthens the hazard identification and RfD derived from the animal data. Nonetheless, we wish to emphasize that, here and in general, human data are not required for the derivation of toxicity values (e.g., RfDs, cancer slope factors). Historically, most toxicity values have been derived from animal data with little or no corresponding human data.

Epidemiology data

Strength of association

"The finding of a large relative risk with narrow confidence intervals strongly suggests that an association is not due to chance, bias, or other factors." (USEPA, 2013)

Table A1 presents the mean estimates, confidence intervals and relationship of the width of the confidence intervals to the mean estimates for those studies that showed statistically significant associations between PFOS exposure and vaccine antibody reduction. The expression, $(|C_1\text{-}C_2|)/\mu$ is analogous to the coefficient of variation. In all but one of the studies (Kielsen et al., 2016), this parameter is $\leq$ 1.5. We interpret this to indicate a relatively narrow confidence interval and suggesting a relatively reliable estimate of the mean. Further, in all but one study (Granum et al., 2013), the mean percent reduction in vaccine antibodies was greater than 10% for the maximum reduction. **We judge the evidence for strength of association as medium-high.**

Table A1
Comparison of mean estimates of vaccine antibody reduction, associated confidence intervals, and the relationship of the confidence intervals to the mean estimates.

Study	Mean estimate of percent reduction in vaccine antibodies (μ)	Confidence interval around mean percent reduction in vaccine antibodies (C_1, C_2)	Relationship of confidence intervals to mean estimates ($\lvert C_1-C_2\rvert)/\mu$
Grandjean et al. (2012)	Tetanus – 29	−46, −6	1.4
(cohort 3)	Diphtheria – 39[a]	− 55, −17	1.0
Grandjean et al. (2017)	Tetanus – N.S.[b]		
(cohort 5)	Diphtheria – N.S		
Grandjean et al. (2017)	Tetanus – N.S.		
(cohorts 3 + 5)	Diphtheria – 24	−37, −10	1.1
Granum et al. (2013)	Rubella – 8	− 14, − 2	1.5
Stein et al. (2016)	Rubella – 13	− 20, − 6	1.1
	Mumps – 6	− 10, − 2	1.3
Kielsen et al. (2016)	Diphtheria – 12	− 22, 0.3	1.8

[a] Largest of two statistically significant associations for diphtheria antibody.
[b] N.S. - Not statistically significant.

Consistency of association

*"An inference of causation is strengthened if elevated risks are observed in independent studies of different populations and exposure scenarios." (*USEPA, 2013*)*

Of the five independent populations investigated, four showed a statistically significant association between a decrease in at least one vaccine antibody and a measure of serum PFOS. One population of adults (Looker et al., 2014) did not show such a relationship (see Table 2). That study was also the only one that investigated only a single vaccine antibody (influenza), and that antibody was not associated with PFOS exposure in the only other study in which the influenza antibody was investigated (i.e., Granum et al., 2013). The Grandjean et al. (2017) Faroe Island cohort (cohort 5) did not show an association between PFOS and either tetanus or diphtheria antibodies, both of which were significantly associated with maternal PFOS exposure (and childhood PFOS exposure for diphtheria antibody) in the earlier Faroes cohort (cohort 3; Grandjean et al., 2012). It seems likely, however that the negative results from cohort 5 were due to the lesser power in that study due to a smaller sample size and a significantly decreased PFOS exposure (approximately one-third the exposure in cohort 3). It should be noted, however, that the combined (3 and 5) cohorts from Grandjean et al. (2017) showed a significant association between PFOS and diphtheria antibody. Among the PFOS-vaccine antibody studies, associations were observed for maternal, childhood and adult exposures. We note, however, that while there were multiple determinations among these studies, there were few repeat determinations of any given vaccine antibody. This limits the ability to evaluate the consistency of association for individual vaccine antibodies. **We judge the evidence for consistency of association as medium.**

Specificity of association

*"As originally intended, this refers to one cause associated with one effect. Current understanding that many agents cause multiple effects and many effects have multiple causes makes this a less informative aspect of causation, unless the effect is rare or unlikely to have multiple causes." (*USEPA, 2013*)*

There are multiple known causes of decreased immune response. In addition, none of the epidemiology studies were able to isolate a PFOS-specific effect from the overall PFAS effect (despite significant associations for PFOS). However, the decreased PFC response in mice that were dosed only with PFOS is analogous to decreased vaccine response. It, therefore, seems reasonable that there is a PFOS-specific association with decreased vaccine antibodies in humans. **We judge the evidence for specificity of association as medium.**

Temporal relationship

*"A causal interpretation requires that exposure precede development of the effect." (*USEPA, 2013*)*

In two prospective birth cohort studies, Grandjean et al. (2012) and Granum et al. (2013) an association was observed between maternal PFOS exposure and childhood vaccine response. Kielsen et al. (2016) was a prospective study in adults that showed an appropriate temporal relationship between vaccination, serum PFOS concentrations and vaccine antibody levels. One of the studies showing a significant negative association between serum PFOS and vaccine antibody levels was a cross-sectional study (Stein et al., 2016) from which temporality cannot be established. **We judge the evidence for existence of a temporal relationship as high.**

Biological gradient (exposure-response relationship)

*"Exposure-response relationships strongly suggest causation. A monotonic increase is not the only pattern consistent with causation. The presence of an exposure-response gradient also weighs against bias and confounding as the source of an association." (*USEPA, 2013*)*

Ideally, the exposure-response relationship would be investigated within each study on the basis of exposure categories (e.g., quartiles of PFOS serum concentration). However, the regression analyses in those studies showing a statistically significant association were not stratified by PFOS concentration. Fig. A1 shows the relationship between the central tendency values for serum PFOS concentrations and the percent reduction in specific vaccine antibodies across the studies showing statistically significant associations. Several caveats must be noted in this comparison. These studies investigated different populations, different vaccine antibodies, PFOS serum concentrations were measured at different ages, and antibody levels were measured at different times after vaccination. Nonetheless, the overall pattern is suggestive of a biological gradient. **We judge the evidence for a biological gradient as low-medium.**

Biological plausibility

*"An inference of causation is strengthened by data demonstrating plausible biologic mechanisms, if available." (*USEPA, 2013*)*

As discussed above, the mode of action (MOA) for suppression of the immune response to foreign antibodies is not known for either reduced vaccine response (humans), or decreased PFC response (animals). The clear demonstration that PFOS causes decreased PFC response in mice is

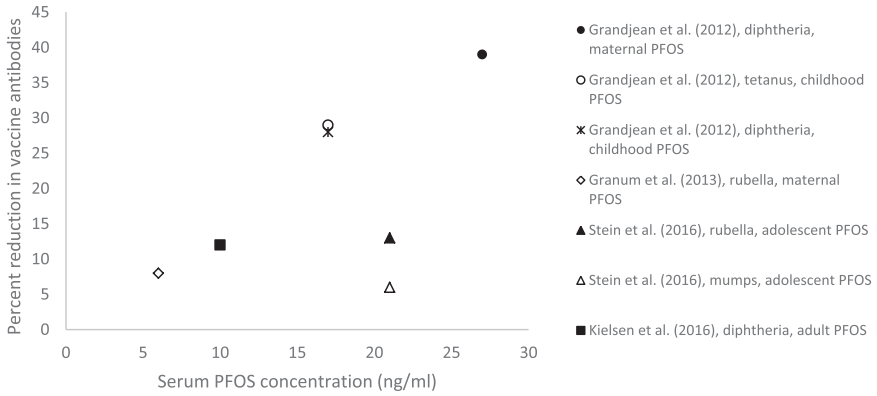

Fig A1. Central tendency values for serum PFOS concentration-vaccine antibody response across epidemiological studies showing statistically significant associations.

highly suggestive that an analogous effect can occur in humans. Lack of definitive mechanistic evidence, however, does not permit consideration of the relevance of a putative animal MOA to the reduced vaccine response in humans. **We judge the evidence for biological plausibility to be low-medium.**

Coherence

*"An inference of causation is strengthened by supportive results from animal experiments, toxicological studies, and short-term tests. Coherence may also be found in other lines of evidence such as changing disease patterns in the population." (*USEPA, 2013*)*

As discussed in detail above, studies in mice provide strong and direct evidence that short-term PFOS exposure can result in decreased antibody response to a foreign antigen. These animal studies are consistent with the four longitudinal cohort studies that show a strong association between gestational PFOS exposure and childhood infections. **We judge the evidence for coherence to be high.**

Natural experiment

*"A change in exposure that brings about a change in disease frequency [or other health-related effect, i.e. vaccine response in this case] provides strong evidence, as it tests the hypothesis of causation." (*USEPA, 2013*)*

Grandjean et al. (2012, 2017) investigated the association between gestational and childhood PFOS exposure and tetanus and diphtheria vaccine antibodies at 5-years old in two birth cohorts (cohorts 3 and 5) in the Faroe Islands. These two cohorts differed notably in their serum PFOS concentrations at 5-years old, with cohort 5 (median = 4.7 ng/ml) having approximately one-third the serum PFOS concentration of cohort 3 (geometric mean = 16.7 ng/ml). The two cohorts also differed with respect to sample size, with the sample size of cohort 5 being 65% of the sample size of cohort 3. The Faroe Islands population is ethnically homogeneous with little population immigration, and no major demographic differences between the two cohorts are likely. The studies do not provide the extensive demographic data that would permit a detailed comparison of the two cohorts and the only obvious reported differences is a longer duration of breastfeeding in cohort 3. While, as discussed, PFOS exposure was significantly associated with decreased tetanus vaccine antibodies at 5-years old in cohort 3 (other associations including those with diphtheria vaccine antibodies were seen with maternal/gestational PFOS exposure and with antibody measurement at 7-years old), no significant association with PFOS exposure were observed for cohort 5. This observation is consistent with a natural experiment of the effects of decreased PFOS exposure on vaccine antibody levels. However, it is also consistent with the reduced sample size of cohort 5. **We judge the evidence for support from a natural experiment to be low-medium.**

Analogy

*"Information on structural analogues or on chemicals that induce similar mechanistic events can provide insight into causation." (*USEPA, 2013*)*

As this assessment focuses specifically on PFOS, it is beyond its scope to review and assess the literature on the immune effects of other PFAS. However, we note that in its review of the immunotoxicity of PFOA and PFOS, NTP (2016) stated that, "…PFOA and PFOS are *presumed to be immune hazards to humans* and to alter immune function in humans. Exposures to PFOA and PFOS are associated with changes in multiple immune outcomes in both experimental animal and epidemiological studies. The strongest bodies of evidence to inform the evaluation of PFOA- and PFOS-associated immunotoxicity are on the antibody response." **We judge the evidence for analogy to be medium-high.**

Animal data

Although focused on investigating causality in epidemiological studies, several of the Hill (1965) criteria are pertinent to animal studies. Strength of response, consistency of response, dose-response relationships, biological plausibility, and coherence can be used to evaluate animal data to determine the potential for effects in humans (USEPA, 2013). For consistency with the above evaluation of epidemiological evidence, the remaining Hill criteria are also applied to the animal evidence.

Strength of response

As shown in Fig. 3, regardless of the range of resulting serum PFOS concentrations among the three studies reporting a decrease in PFC response following PFOS exposure in adult animals (Peden-Adams et al., 2008; Dong et al., 2009; Zheng et al., 2009), the LOAEL serum PFOS concentrations corresponded to a 30–60% reduction in PFC response in males in each of the studies. Further, at the highest serum PFOS concentrations, a PFC response reduction in males ranged from 69% to 86%. **We judge the evidence for strength of response among these studies to be high.**

Consistency of response

We identified five studies that investigated the effect of PFOS on PFC response in mice. No such studies were identified in other species. Four of these studies showed a statistically significant decrease in PFC response. The single negative study (Qazi et al., 2010) differed in some respects from the other studies in using a dietary (as opposed to gavage) route of exposure, and in using the tetraethylammonium (as opposed to potassium) salt of PFOS. It is not clear to what extent these differences may account for the different results observed in this study. The positive results among these studies were observed in two different strains of mice (C57BL/6 and B6C3F1), in males and females (although males were more sensitive) and with exposure at two different life-stages (adult and gestation [Keil et al., 2008]). **We judge the evidence for consistency to be medium-high.**

Specificity of response

As discussed above, all but one study reported a decrease in PFC response following PFOS exposure. As PFOS was the sole intended toxicant used for exposure, and controls and dosed animals were maintained under similar conditions, there can be little doubt that a decrease in PFC response resulted from PFOS exposure. **We judge the evidence for specificity of response as high.**

Temporal relationship

In all four mouse studies that reported a decrease in PFC response, PFOS exposure preceded the assessment of this endpoint. **We judge the evidence for a temporal relationship as high.**

Dose-response relationships

The four studies with positive results had monotonic dose-response relationships with a trend of decreasing PFC response with increasing PFOS serum concentration. In the Peden-Adams et al. (2008) study, the three highest PFOS doses gave an essentially flat response in males. However, as the serum PFOS concentrations corresponding to these doses were not reported, it is not known how this is reflected in terms of serum PFOS concentration. In all other positive studies, the decrease in PFC response was continuous across all serum PFOS concentrations. For the three positive studies following adult exposures that reported serum PFOS concentration (Peden-Adams et al., 2008; Dong et al., 2009; and Zheng et al., 2009), the LOAELs differed widely with a range of 92–1.1 $\times$ 10^5 ng/ml and more than an order of magnitude between the LOAELs of any two of the studies. As discussed above, this may be due to several factors including, different degrees of antigenicity among different sources of sheep RBCs, differences in baseline PFC response among the control mice in each study, and different serum PFOS concentrations in control mice. **We judge the evidence for the *existence* of a dose-response/serum-concentration-response to be high. We judge the evidence for the *consistency* of the serum-concentration relationship among these studies to be low.**

Biological plausibility

As above, there is no clear MOA for either decreased PFC response in mice or decreased vaccine antibody response in humans. However, given the demonstration of decreased PFC response in four different mouse studies, with failure to observe this effect in only one study, there appears to be little reason to doubt that this effect does, in fact, occur in mice in response to PFOS administration. In this case, the absence of a known MOA does not reduce the empirical plausibility of the observed response. **We judge the evidence for biological plausibility to be high.**

Coherence

As discussed previously, the coherence of the decreased PFC response in mice resulting from PFOS administration is attested to by qualitatively similar results from four different studies with only a single study failing to show this response. In addition, decreased vaccine response in humans is directly analogous to decreased PFC response. There was an association between PFOS exposure and decreased vaccine antibody response in four different human populations, with only single study failing to show such an association. Furthermore, there is an observation of an association between PFOS exposure and increased childhood infections in four different populations. These observations, in total, provides strong evidence for the coherence of immune suppression in animals and humans. **We judge the evidence for coherence to be high.**

Natural experiment

This criterion is not applicable to designed and controlled animal studies.

Analogy

An in-depth review of immune effects of PFOA in experimental animals is beyond the scope of this discussion. However, we note that in its review of the immunotoxicity of PFOA and PFOS, NTP (2016) stated that, “There is high confidence that exposure to PFOA is associated with suppression of the antibody response in animals based on consistent suppression of the primary antibody response from experimental studies in mice.” In addition, perfluorononanoic acid (PFNA), which like PFOS, has eight fully fluorinated carbons, also exhibits immunotoxicity in rodents (Fang et al., 2008, 2009, 2010). **We judge the evidence for analogy to be high.**

Integrative assessment of the weight of evidence

Table A2 summarizes the strength of the conclusion underlying the RfD that PFOS-mediated suppression of antibody response is a relevant human health endpoint for environmental exposure to PFOS.

This integrative assessment aims to evaluate the overall weight of evidence that PFOS-mediated suppression of antibody response to foreign antigens is a relevant human health endpoint for environmental exposure to PFOS. However, there is no straightforward approach for counting or averaging the individual evaluations to produce a summary evaluation of the weight of evidence. This is because some criteria are more directly relevant to this question than others.

We judge that the weight of evidence from the epidemiology studies is overall medium. Much of the residual weakness in the weight of evidence from these studies is due to the lack of confirmatory studies for individual vaccine antibodies, lack of evidence for a MOA, and lack of data from which to evaluate exposure-response within individual studies, as well as the inability to identify a unique PFOS association from within the overall database of PFAS effects on vaccine antibody suppression. With respect to the last of these, it should be noted that a PFOS-specific effect is clearly seen in the animal studies.

Table A2
Summary of weight of evidence conclusions based on epidemiologic and animal evidence.

Criterion	Assessment of evidence
Epidemiologic data	
Strength of association	Medium-high
Consistency of association	Medium
Specificity of association	Medium
Temporal relationship	High
Biological gradient (exposure-response relationship)	Low-medium
Biological plausibility	Low-medium
Coherence	High
Natural experiment	Low-medium
Analogy	Medium-high
Animal data	
Strength of response	High
Consistency of response	Medium-high
Specificity of response	High
Temporal relationship	High
Dose-response relationships	
- Existence of a serum-concentration relationship	High
- Consistency of the serum-concentration relationship among studies	Low
Biological plausibility	High
Coherence	High
Natural experiment	Not relevant to animal studies
Analogy	High

We judge that the weight of evidence from the animal studies is overall medium-high to high. This is attributed to the strength, consistency, plausibility, and coherence of decreased PFC response in animals. The primary weakness in the animal data is the lack of consistency in quantitative metrics (e.g., LOAELs) of dose-response (serum concentration-response) across studies.

Overall, we judge the weight of evidence that suppression of antibody response is a relevant human health endpoint for environmental exposure to PFOS to be medium-high.

Appendix C. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envres.2018.08.004.

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