NTP Evaluation Concept: Immunotoxicity Associated with Exposure to PFOA or PFOS

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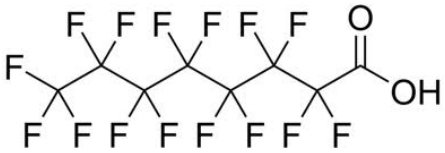
Background and Rationale

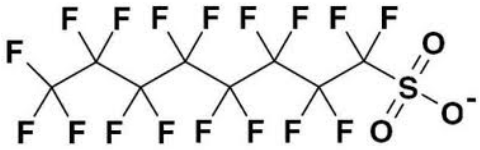
Nomination history

The Office of Health Assessment and Translation (OHAT) developed 2 case study evaluations to test the OHAT framework for systematic review and evidence integration, one of which was an evaluation of the association between exposure to perfluorooctanoic acid (PFOA) or perfluorooctane sulfonate (PFOS) and immunotoxicity (http://ntp.niehs.nih.gov/go/36501; structures presented in Figure 1). The case study was used to provide input for refining the OHAT framework, and was not intended to result in hazard identification conclusions. Although a detailed protocol was developed and peer-reviewed to outline the approach for conducting the evaluation, only subsets of the studies were used for any step in the process because the goal was to test the systematic review procedures and avoid issues with the specifics of the case study. The case-study phase was completed and the OHAT framework for systematic review and evidence integration for literature-based health science evaluations was published earlier this year (Rooney *et al.* 2014). During the case-study process, we received multiple requests to complete the PFOA and PFOS case study as a full OHAT evaluation with the goal of reaching an immunotoxicity hazard identification conclusion. This evaluation topic was also considered in conjunction with the nomination and ongoing assessment of perfluorinated compounds including PFOA and PFOS by NTP's testing program. Additional immunotoxicology testing is not currently suggested for either PFOA or PFOS because there are sufficient published experimental studies of immune effects for both chemicals. Therefore, NTP proposes to conduct a literature-based evaluation focused on immune-related health effects that would be complementary to the ongoing assessment of perfluorinated chemicals by the NTP's testing program.

Figure 1: Structure of PFOA and PFOS



perfluorooctanoic acid (PFOA)



perfluorooctane sulfonate (PFOS)

Background

PFOA and PFOS are extremely persistent chemicals that are widely distributed in the environment in part because of high stability and little to no expected degradation in the environment (Lau *et al.* 2007, EFSA 2008, ATSDR 2009, US EPA 2014b). Once in surface water, apparent half-lives of PFOS and PFOA are 41 and 92 years respectively under typical environmental conditions. Estimated half-lives in the human body are also long, ranging from 2 to 8 years (ATSDR 2009, Steenland *et al.* 2010, US EPA 2014b). In terms of toxicity and exposure, PFOA and PFOS are the best studied perfluoroalkyl acids, a group of compounds used extensively over the last 50 years in commercial and industrial applications including food packaging, lubricants, water-resistant coatings, and fire-retarding foams. Through voluntary

agreements, the primary manufacturer of PFOS phased out production in 2002 and PFOS is no longer manufactured in the United States (US EPA 2006, ATSDR 2009, US EPA 2009). Similar arrangements have been made for PFOA and eight companies that manufacture PFOA have committed to eliminate emissions and product content by 2015 (US EPA 2006, ATSDR 2009, US EPA 2013, 2014b).

Although emissions have been dramatically reduced, the persistence and bioaccumulation of both PFOA and PFOS result in detectable levels in the U.S. population and therefore are of potential human health relevance (US EPA 2014b). PFOA and PFOS were present in all of the 1562 serum samples analyzed as part of a study of 11 perfluorinated compounds in the National Health and Nutrition Examination Survey (NHANES 1999-2000) (Calafat *et al.* 2007) and remain the two highest concentrations among perfluorinated compounds measured in blood from the general U.S. population in the most recent National Report on Human Exposure to Environmental Chemicals for 2009-2010 (CDC 2014).

Several recent publications from 2012-2014 have linked PFOA and PFOS exposure to functional immune changes in humans that are consistent with evidence of PFOA- and PFOS-related immunotoxicity in animal studies. Immune-related health effects including suppression of the antibody response to vaccines and increased incidence of autoimmune ulcerative colitis have been reported in adults living in an area of Ohio and West Virginia where public drinking water had been contaminated with PFOA (Steenland *et al.* 2013, Looker *et al.* 2014). PFOA- and PFOS-associated antibody suppression were also described in prospective cohort studies of children in Norway (Granum *et al.* 2013) and the Faroe Islands (Grandjean *et al.* 2012).

Suppression of the antibody response in mice has been reported at blood concentrations of PFOS occurring in the general U.S. population (e.g., Peden-Adams *et al.* 2008, Fair *et al.* 2011, DeWitt *et al.* 2012, CDC 2013). Experimental studies of PFOA and PFOS in laboratory animals have also demonstrated exposure-related suppression of the antibody response among other immune changes including altered inflammatory response, cytokine signaling, and measures of both innate and adaptive immunity (reviewed in DeWitt *et al.* 2012). Wildlife studies in species ranging from loggerhead sea turtles to sea otters have also reported widespread exposure and altered immune measures associated with PFOA and PFOS (e.g., Keller *et al.* 2005, Kannan *et al.* 2006, Hart *et al.* 2009).

Rationale

OHAT proposes to examine the evidence that exposure to PFOA or PFOS is associated with immunotoxicity or immune-related health effects. The immune effects observed in experimental animals have been reported at the lower end of the dose range among health effects observed, with immune effects at 0.49mg PFOA/kg/day (Son *et al.* 2009) and 0.00166mg PFOS/kg/day (Peden-Adams *et al.* 2008). These doses are similar to, or lower than, levels associated with the most sensitive endpoints such as increased liver weight and developmental toxicity (ATSDR 2009, US EPA 2014c, d). Given the recent publication of human studies with functional immune effects linked to PFOA and PFOS, the timing is good to evaluate this emerging evidence along with the previous animal studies. The proposed evaluation would also leverage the preliminary work done in this area when subsets of the available evidence were assessed as part of a case-study to test the OHAT framework for systematic review and evidence integration (e.g., see http://ntp.niehs.nih.gov/go/36501 for the evaluation protocol).

To our knowledge, there is no published systematic review on this topic, nor one currently under development. The US Environmental Protection Agency (EPA) periodically releases an "emerging contaminant" fact sheet on PFOA and PFOS (last updated March 2014) that briefly outlines chemical properties, additional sources of information, federal and state guidelines, and lists several potential health impacts. To date, these fact sheets have not included statements on immune effects (US EPA 2014b). Several reviews have concluded that PFOA and PFOS are immunotoxicants based primarily on the animal evidence (e.g., DeWitt *et al.* 2012). There are several draft hazard assessments (US EPA 2005,

ATSDR 2009, US EPA 2014c, d) that have described PFOA and PFOS-related immune effects; however, these assessments also primarily focus on experimental animal data because they were prepared before (or used literature search cut-off dates prior to) the publication of recent human studies with data on functional immune measures. Only one of the three studies reporting suppressed antibody response to vaccination was available for previous evaluations (Grandjean *et al.* 2012, Granum *et al.* 2013, Looker *et al.* 2014). Similarly, the ulcerative colitis data had not been published and only one of the osteoporosis reports had been released at the time the draft hazard assessments were developed (Innes *et al.* 2011, Steenland *et al.* 2013, Uhl *et al.* 2013).

Given the extent of the human and animal evidence observed during scoping and the case study process (particularly on the antibody response), it is anticipated that the proposed evaluation of PFOA- and PFOS-related immunotoxicity would result in hazard conclusions. This information could benefit other agencies, especially since the OHAT evaluation would be conducted using systematic review methods and the data extraction could be shared including individual study quality/internal validity assessment. The Agency for Toxic Substances and Disease Registry (ATSDR) and EPA's Office of Water and Office of Pesticide Prevention and Toxics have ongoing assessments of health effects of PFOA and PFOS (US EPA 2013, 2014a). The NTP has contacted the assessment mangers for these chemicals at ATSDR and EPA, and will maintain communication to aid in the potential utility of the assessment.

Scoping and Problem Formulation

Although the OHAT case-study evaluation of PFOA and PFOS and immunotoxicity was not intended to result in hazard identification conclusions, a detailed protocol was developed for the evaluation including specifics of the search strategy, eligibility criteria for studies considered in the review, and procedures used for each step in the evaluation. During the scoping and problem formulation process for the case study government agencies including the EPA and ATSDR were consulted because of interest and expertise in toxicity issues of the perfluoroalkyl acids. The protocol and literature search strategy were reviewed by technical experts with backgrounds in immunotoxicology, PFOA and PFOS, and systematic review; distributed to other government agencies through the NTP executive committee and points of contact; and revised based on comments received. The scope and focus were outlined in the protocol which was posted for public comment in April of 2013 (http://ntp.niehs.nih.gov/go/36501). The literature search strategy for the case study and the inclusion and exclusion criteria are included in supplemental materials. The extent of the database of relevant studies for the case study is described below.

Overview of scientific information regarding immunotoxicity

The literature search for the case study was conducted in June of 2013. At that time, over 2,500 studies were retrieved, of which 114 studies were judged to be relevant to the evaluation following screening. The search is currently in the process of being updated and we expect to add additional studies that were published in 2013 and 2014. The list of relevant studies included 18 human studies, 80 animal studies, and 19 mechanistic studies (3 studies had both animal and mechanistic data). The animal studies were primarily experimental, controlled-exposure studies in rodents; however, there were 5 observational or wildlife studies. While the antibody response data were highlighted in the background and the focus of the subset of data that were explored as part of the case study, there are other immune data that will also be examined as part of the evaluation. Human data include reported links to inflammation or autoimmune-related health effects such as ulcerative colitis and osteoporosis (Steenland *et al.* 2013, Looker *et al.* 2014, Woodruff and Sutton 2014). Animal data also include altered inflammatory response and cytokine signaling, as well as other functional measures such as natural killer cell response (Peden-Adams *et al.* 2008, Matcher 2012) or observational endpoints such as lymphocyte proliferation (Samson and Schoeles 2012).

Issues and Key Scientific Questions

There are several scientific questions anticipated for this evaluation: (1) the consideration of developing conclusions across the two chemicals; (2) the relevance of peroxisome proliferator-activated receptor alpha (PPARα) as a mechanism for immune effects and species differences between animal models and humans; and (3) the importance of pronounced species differences in elimination rates for PFOA and PFOS between experimental animals and humans.

NTP plans to develop conclusions separately for PFOA and for PFOS. The evidence on specific health effects will then be compared between the two chemicals when there are data on the same or related immune effects. For example, the database for PFOA and PFOS both include data on the antibody response, and the evidence for effects will be compared between the two chemicals. We are not planning a mixtures assessment or a statement regarding the immunotoxicity of the related class of perfluoroalkyl acids based on this evidence alone.

The role of PPARα in the mechanism for immune effects will be considered when evaluating the animal immune data because of strong species differences in PPARα between rodents and humans. Some of the health effects observed in experimental animals have been linked to the ability of PFOA and PFOS to activate the peroxisome proliferator-activated receptor alpha (PPARα), and others have been shown to be independent of PPARα. For example, developmental effects of PFOA including neonatal lethality were shown to be PPARα-dependent (Abbott *et al.* 2007), while PFOS induced neonatal lethality and delayed eye opening that was independent of PPARα (Abbott *et al.* 2009). The mechanism of action for immune effects of PFOA and PFOS are not understood at this time. Targeted studies suggest that immune effects reported in laboratory animals appear to be partially or wholly independent of PPARα (DeWitt *et al.* 2009, DeWitt *et al.* 2012). This is particularly the case for suppression of the antibody response for which there is evidence that PFOA- and PFOS-associated suppression in mice are not dependent on PPARα (reviewed in DeWitt *et al.* 2012) and there are human data on PFOA- and PFOS-associated suppression in antibody response to vaccination. Studies conducted in mice, rats, and other mammalian model systems will be considered relevant for humans unless compelling evidence to the contrary is identified during the course of the evaluation.

Species differences in elimination rates are important when considering dose level used in experimental animals studies. Although there is little evidence for gender differences in elimination rates in humans or non-human primates, there are gender and age differences in elimination rates in rodents (e.g., male rats have lower rates than females). Known, species, gender, and age differences in elimination will be considered in evaluating the consistency of results reported for a given health effect. NTP recognizes that the dose or level of exposure is an important factor when considering the relevance of study findings. In the OHAT evaluation process, consideration of dose would occur after hazard identification as part of reaching a "level of concern" conclusion when the health effect is interpreted in the context of what is known regarding the extent and nature of human exposure (Shelby 2005). PFOA and PFOS have significantly lower elimination rates in humans than experimental animals resulting in long half-life values in humans (2-8 years) compared to half-life values from 10 to 20 days in monkeys and rodents (ATSDR 2009). The significantly slower elimination rates of PFOA and PFOS in humans compared to experimental animals would require pharmacokinetic adjustment to evaluate effective doses for immune effects in humans based on experimental animal evidence.

Specific Aims

The overall objective is to develop hazard identification conclusions that exposure to PFOA or PFOS is associated with immunotoxicity or immune-related health effects. If the database is sufficient, we intend to develop hazard conclusions. If the data are insufficient, we will develop a state of the science

report. The objective will be addressed by answering the key questions (KQ) for the proposed evaluation as outlined in Table 1. Although PFOA and PFOS are both considered in this evaluation, conclusions will be developed separately for each chemical. The available studies for each of the three evidence streams (human, animal, and mechanistic or other relevant studies) will be evaluated separately to address the key questions. Then, hazard identification conclusions of "known", "presumed", "suspected", or "not classifiable" to be associated with immunotoxicity will be developed for PFOA and PFOS by integrating the human and animal evidence with consideration of the impact of mechanistic or other relevant data.

Table 1: Key Questions (KQ)	
	What is our confidence in the body of evidence from human studies for the association between exposure to PFOA or PFOS and immunotoxicity or immune-related health effects?
	What is our confidence in the body of evidence from animal studies for the association between exposure to PFOA or PFOS and immunotoxicity or immune-related health effects?
	How does the evidence from other relevant studies (e.g., mechanistic or *in vitro* studies) support or refute the biological plausibility of the association between exposure to PFOA or PFOS and immunotoxicity or immune-related health effects?

Proposed Approach

OHAT has formed an evaluation design team of NIEHS and NTP scientists with expertise on immunotoxicity and/or PFOA and PFOS as we further refine the focus and objectives for this project. In addition to discussions with NIEHS/NTP scientists with relevant expertise, OHAT has solicited input from scientists at other federal agencies with experience on evaluating health effects of PFOA or PFOS as well as immunotoxicity or immune-related health effects including scientists at the EPA and the National Institute for Occupational Safety and Health (NIOSH).

The evidence that PFOA or PFOS exposure is associated with immunotoxicity or immune-related health effects will be evaluated using the OHAT approach to systematic review and evidence integration (http://ntp.niehs.nih.gov/go/38673; (Rooney *et al.* 2014)). Given that an evaluation of PFOA and PFOS exposure an immunotoxicity was used as a case study to test the OHAT methods, a draft protocol for that evaluation was posted on the OHAT website (http://ntp.niehs.nih.gov/go/36501). The draft protocol and search strategy are expected to be used as the basis for the protocol for this evaluation with input and modifications from the evaluation design team, public comments received during the case-study process, and to reflect current OHAT practices. After additional steps to refine the project, the protocol will be posted and other key milestones in the evaluation will be announced on the NTP listserv (e.g., posting list of included studies). Data management will be conducted in a manner that permits public sharing of the literature search results as well as data extracted from studies in a database format when the monograph is finalized following peer-review. Sharing of data in this format should facilitate future updates to this evaluation conducted by NTP or other organizations.

Significance

The proposed evaluation is anticipated to reach hazard identification conclusions for PFOA and PFOS-associated immunotoxicity. The NTP evaluation would not only provide an immunotoxicity hazard assessment that would include an evaluation of the recent human functional immune health effect data, but the evaluation would be conducted using systematic review methods. Therefore, the evaluation would include assessment of individual study quality/internal validity and the data extraction files can be shared with the public and other agencies. The Agency for Toxic Substances and Disease Registry

(ATSDR) and EPA's Office of Water and Office of Pesticide Prevention and Toxics have ongoing assessments of health effects of PFOA and PFOS (US EPA 2013, 2014a). The NTP has contacted the assessment mangers for these chemicals at ATSDR and EPA, and will maintain communication to aid in the potential utility of the assessment.

The immunotoxicity data on PFOA and PFOS for common endpoint (e.g., antibody-related effects) in humans and animals may provide an opportunity for exploring techniques to use and assess mechanistic data such as high throughput assays in ToxCast and Tox21. After the hazard identification conclusions are developed for PFOA and PFOS, OHAT will consider the available toxicity, mechanistic, *in vitro*, and high throughput data related to immunotoxicity of other perfluoroalkyl acids. A determination will be made at that time as to whether the PFOA and PFOS data may lay the ground work for an evaluation of predicative ability of these mechanistic data. Potential questions include whether or not the hazard ID conclusions for PFOA and PFOS could have been predicted from the available mechanistic data, can predictions be made on the immunotoxicity associated with exposure to perfluoroalkyl acids in general. Could the combined data sets be used to explore methods of using the relatively well studied PFOA and PFOS data (with human, animal, and mechanistic studies) along with mechanistic or *in vitro* data on other perfluoroalkyl acids to evaluate the potential immunotoxicity hazard of perfluoroalkyl chemicals that are data poor.

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SUPPLEMENTAL MATERIAL

Draft eligibility criteria for considering studies based on the case study

Types of studies

There are no restrictions based on study design.

Types of human studies and model systems

Studies of humans, animals (experimental and wildlife [e.g., observational animal studies]), or *in vitro* model systems of immune endpoints are considered relevant. There are no restrictions based on lifestage at exposure or assessment, sex, animal species or strain, or immune model system.

Types of exposures

Exposure to PFOA (CAS# 335-67-1) and PFOS (CAS# 1763-23-1) based on administered dose or concentration, biomonitoring data (e.g., urine, blood, or other specimens), environmental measures (e.g., air, water levels), or indirect measures such as job title. There will be no exclusions based on the analytical method (or indirect measure) used to measure PFOA or PFOS, differences in the sensitivities of these methods will be considered when assessing internal validity or risk of bias of individual studies.

Types of outcomes

Immunotoxicity considered in this evaluation is defined in the context of immune responses and changes in immune-related measures that reflect the four main categories of immune response: immunosuppression, immunostimulation, sensitization and allergic response, and autoimmunity. Publications must include an indicator of PFOA or PFOS exposure analyzed in relation to any one of the following primary or secondary outcomes listed in **Table S1** for human and animal studies. Primary outcomes are considered to be the most direct, or applicable, to the project. Secondary outcomes are relevant, but less direct and can include upstream indicators, risk factors, intermediate outcomes, or related measures to our primary outcomes.

For the evaluation of immunotoxicity, primary outcomes are those with more predictive value for immunotoxicity such as disease resistance assays and functional immune parameters. Secondary outcomes are those with less predictive value for immunotoxicity such as observational parameters including cell counts or cytokine levels. This dichotomy separating the more and less predictive measures of immunotoxicity is consistent with testing strategies that rely on more sensitive and predictive immune assays (see Luster *et al.* 1992, US EPA 1996a, b, 1998) and the NTP and WHO methods to categorize the evidence of immune system toxicity. Under these systems, measures of immune function or the ability of the immune system to respond to a challenge are weighed more heavily than observational parameters (Germolec 2009, WHO 2012).

For *in vitro* studies, we are interested in immune measures that may support the biological plausibility of observed immune outcomes. For example, *in vitro* stimulation of immunoglobulin E (IgE) production would support a functional measure of sensitization or allergic response, but it would not support suppression of the natural killer (NK) response. It is generally accepted that *in vitro* systems to evaluate sensitization or immunosuppression would not be able to reproduce the complexity of cellular and soluble interactions that are involved in immune response (this is not unique to the evaluation of immunotoxicity). However, tiered approaches for *in vitro* assays have been proposed and progress has been made in developing assays or groups of assays to assess immunotoxicity with *in vitro* tests (Gennari *et al.* 2005, Carfi *et al.* 2007, Galbiati *et al.* 2010, Lankveld *et al.* 2010). Given the complexity of the immune response, the *in vitro* assessment of immunotoxicity is more likely to have predictive value when the substance evaluated is a direct immunotoxicant, such as a chemical that displays myelotoxicity (killing of immune cells).

Table S2. Outcomes considered relevant for study eligibility

Immune-related diseases and measures of immune function *Immunosuppression* (e.g., otitis, infections, or decreased vaccine antibody response); *Sensitization and allergic response* (e.g., atopic dermatitis or asthma); *Autoimmunity* (e.g., thyroiditis or systemic lupus erythematosus)	Disease resistance assay or measures of immune function *Disease resistance assays* (e.g., host resistance to influenza A or trichinella, changes in incidence or progression in animal models of autoimmune disease) *Immune function assays following <u>in vivo exposure</u> to the test substance* (e.g., antibody response [T-cell dependent IgM antibody response (TDAR)], natural killer cell [NK] activity, delayed-type hypersensitivity [DTH] response, phagocytosis by monocytes, local lymph-node assay [LLNA])	*Immune function assays following <u>in vitro exposure</u> to the test substance* (e.g., natural killer cell [NK] activity, phagocytosis or bacterial killing by monocytes, proliferation following anti-CD3 antibody stimulation of spleen cells or lymphocytes)
*Immunostimulation*** (e.g., unintended stimulation of humoral immune function) *Observational immune endpoints* (e.g., lymphocyte counts, lymphocyte proliferation, cytokine levels, serum antibody levels, or serum autoantibody levels)	*Observational immune endpoints* (e.g., lymphoid organ weight, lymphocyte counts or subpopulations, lymphocyte proliferation, cytokine production, serum antibody levels, serum or tissue autoantibody levels, or histopathological changes in immune organs)	*Observational immune endpoints following <u>in vitro exposure</u> to the test substance* (e.g., general mitogen-stimulated lymphocyte proliferation, cytokine production)

* Note that the protocol will consider experimental animal studies and observational animal studies (e.g., wildlife studies without a controlled exposure).

** Note that stimulation of the immune response is not adverse per se and most vaccine preparations include adjuvants to aid in stimulation of an immune response to microbes. It is generally agreed that stimulation of the immune system should not be disregarded (WHO 2012). Unintended immunostimulation will be considered for possible hazard in the context of potency and persistence of the elevated immune response. Because evaluation of immunostimulation is less well established for health assessment, outcomes that could be evaluated under autoimmunity or sensitization will be evaluated under these more established categories when possible.

Currently within the field of immunotoxicology, *in vitro* data in the absence of *in vivo* human or animal data are considered to provide evidence that is of low predictive value for hazard identification conclusions. *In vitro* approaches play a role as a screening tool to identify chemicals that should be subjected to more predictive immunotoxicity testing (Galbiati *et al.* 2010, WHO 2012). In the context of

this evaluation, it is envisioned that strong evidence for a relevant immune process from mechanistic or *in vitro* data alone could indicate a greater potential that the substance is an immune hazard to humans and *in vivo* studies are suggested for a more definitive conclusion.

Types of publications

Publications must be peer-reviewed articles or meet the guidelines for hand selection or grey literature used by OHAT. There are no date or language restrictions. Review articles and health assessments will be collected for the purposes of reviewing the reference list and will not contribute to the final number of studies considered eligible unless they contain original data.