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***In vitro* evaluation of the effects of perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) on IL-2 production in human T-cells**

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Abstract

Perfluorinated compounds, such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), have been shown to alter various immune functions suggesting they are immunotoxic. This study assessed the effects of PFOS and PFOA on interleukin (IL)-2 production in the human Jurkat T-cell line and PFOS in healthy human primary T cells. Jurkat cells were stimulated with phytohemagglutinin (PHA)/phorbol myristate acetate (PMA), anti CD-3/anti CD-28, or anti CD-3, and dosed with 0, 0.05, 0.1, 0.5, 1, 5, 10, 50, 75, or 100 $\mu\text{g ml}^{-1}$ PFOS or 0, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, or 10 $\mu\text{g ml}^{-1}$ PFOA. Jurkat cells stimulated with PHA/PMA or anti CD-3 exhibited decreased IL-2 production beginning at 50 $\mu\text{g PFOS ml}^{-1}$ and 5 $\mu\text{g PFOS ml}^{-1}$ respectively, but stimulation with anti-CD3/anti-CD28 resulted in no changes compared with the control. Addition of the PPAR-alpha antagonist GW6471 to PFOS-dosed cells stimulated with PHA/PMA resulted in decreases in IL-2 production starting at 50 $\mu\text{g PFOS ml}^{-1}$, which suggests PFOS affected T-cell IL-2 production via PPAR-alpha-independent mechanisms. Exposure to PFOA, PFOA + GW6471, or PFOS + PFOA in Jurkat cells resulted in no significant differences in IL-2 production. *In vitro* dosing studies using healthy primary human CD4⁺ T cells were consistent with the Jurkat results. These data demonstrated that PFOA did not impact IL-2 production, but PFOS suppressed IL-2 production in both a human cell line and human primary cells at dose levels within the high end of the human exposure range. A decrease in IL-2 production is characteristic of autoimmune diseases such as systemic lupus erythematosus and should be further investigated.

Keywords

PFOS; PFOA; IL-2 production; immunology; ppar-alpha; perfluorinated compounds; cytokine; human T cells; in vitro; immunosuppression

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Conflict of Interest

The Authors did not report any conflict of interest.

Introduction

The potential human health effects of perfluorinated compounds such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are becoming an increasing concern in the United States and worldwide. PFOS and PFOA are part of an emerging class of contaminants known as perfluoroalkyl acids (PFAAs). PFOS is a surfactant and historically has been used in a variety of products including surface treatments, paper production, and in performance chemicals such as firefighting foams, floor polishes, photographic film, denture cleaners, shampoos, carpet spot cleaners and as an insecticide in bait stations (OECD, 2002). PFOA is used in manufacturing of non-stick cookware and stain repellent carpet treatments. Monitoring data indicate that the general population may be exposed to PFOS and PFOA via ingestion of contaminated fish, drinking water, and dermal contact with products containing the chemicals (EPA, 2013).

PFAAs are carbon chains that have had the hydrogens replaced with fluorine and they contain various substitution groups (R-groups) on the terminal end that impart their active properties. This substitution with fluorine creates carbon–fluorine bonds that are extremely strong making these compounds very stable in the environment and in the body (Giesy *et al.*, 2001). PFAAs are resistant to typical environmental degradation processes and are, therefore, widely distributed and found in water, soil, and air at sites around the United States. There was enough concern over these compounds that the USEPA nominated PFOS and related fluorochemicals to CDC for inclusion in the National Health and Nutrition Examination Survey (NHANES) in 2003 (EPA, 2003). PFAA plasma concentrations were increasing in adult and children human blood samples in the United States until 2004 (Olsen *et al.*, 1999, 2001a, 2001b, 2003a, 2003b; Harada *et al.*, 2004). Of all the perfluorinated compounds, PFOS is the one that is found at the highest concentration in blood serum (PFOA is found at the second highest concentration). In 2000, 3M, the primary American producer of PFOS, announced the phase-out of PFOS and PFOS-related products in response to studies related to the toxicity of PFOS (3M, 2008). PFOA is still being manufactured, but in accordance with the 2010/2015 PFOA Stewardship Program, the eight major fluoropolymer and telomer manufacturers committed to achieve a 95% reduction in PFOA by 2010 and to work towards the elimination of these PFOA and PFOA breakdown products by 2015 (EPA, 2014). PFOS is still detected in the serum of almost all people in the United States, but levels are starting to decrease since the phase-out (Renner, 2008). However, PFOS levels in blood serum in China continue to increase where PFOS and PFOS-related compounds are still being manufactured (Renner, 2008). In addition, a recent study in Dallas children showed perfluorinated compound levels increasing from birth to 12 years of age in spite of discontinued and reduced manufacturing of PFOS and PFOA, respectively (Schechter *et al.*, 2012). These children are still being exposed to perfluorinated compounds years after changes in manufacturing. In addition to these effects, cause for concern about PFOS and PFOA is as a result of the long half-lives of these compounds. In humans, PFOS has an average half-life 5.4 years and PFOA has an average half-life of 2.3 years (Olsen *et al.*, 2007; Bartell *et al.*, 2010). Between 1999–2008, mean serum levels in humans 12 years of age in the United States are reported at 20.6 ng ml⁻¹ for PFOS and 4.4 ng ml⁻¹ for PFOA (Kato *et al.*, 2011).

Although little data are available on the toxicity of many perfluorinated compounds, much is known about PFOS and PFOA. These compounds both cause peroxisomal proliferation and liver damage, alter estradiol and thyroid hormone pathways and have health effects related to genotoxicity, reproductive and developmental toxicity, and carcinogenicity (Kennedy *et al.*, 2004; Lau *et al.*, 2007). In addition to all of these effects, both PFOS and PFOA have been shown to alter various immune functions in mice suggesting that they are immunotoxic. A 28-day oral exposure to PFOS in adult B6C3F1 female mice resulted in increased *ex vivo* basal production of interleukin (IL)-6 from B-cell and decreased basal production of IL-2 by T-cells (Fair *et al.* 2011; Peden-Adams *et al.*, 2011; DeWitt *et al.*, 2012,). PFOA (0.02%) was added to the feed of male C57B1/6 mice for 7–10 days and caused a reduced body weight, reduced numbers of thymus and spleen cells, reduced numbers of CD4+ and CD8+ cells in the thymus, an increase in liver weight, and peroxisome proliferation (Yang *et al.*, 2002a, 2002b). PFOS and PFOA exposure have been shown to decrease T-cell-dependent IgM antibody responses in mice (Dewitt *et al.*, 2008, Peden-Adams *et al.*, 2008). PFOS also decreased T-independent IgM production and decreased host resistance to influenza A (Peden-Adams *et al.*, 2008; Guruge *et al.*, 2009). These immune effects have recently been seen in human studies as well. Grandjean *et al.* (2012) showed that in children between the ages of 5 and 7 years old, PFOS and PFOA concentrations in serum at commonly seen levels is associated with lower antibody responses to childhood immunizations such as diphtheria and tetanus. This is one of the first studies to demonstrate childhood deficits in immune system functions connected to exposure of PFOS and PFOA.

IL-2 is a very important cytokine as it is required for generation and maintenance of regulatory T cells (Tregs), which are needed to provide lifelong protection from autoimmune disease (Malek, 2003). A decrease in T cell IL-2 production was seen after PFOS exposure in two different studies. Dong *et al.* (2011) showed decreases in numbers of T-cells secreting IL-2 (ELISPOT) at 50 mg kg⁻¹ total administered dose (TAD) over 60 days and Zheng *et al.* (2011) showed decreased numbers of T-cells producing IL-2 after a 7-day exposure to 20 mg kg⁻¹ day⁻¹ PFOS (both in male C57B1/6 mice). These same studies also showed that after both short-term and subchronic PFOS exposure, the cytokine balance favored T-helper (Th)-2 responses (Dong *et al.*, 2011, Zheng *et al.*, 2011). PFOA has not been previously investigated for modulation of IL-2 production.

The current study assessed the effects of PFOS and PFOA on IL-2 production in the human Jurkat T-cell line and primary human cells. Because a decrease in IL-2 production was seen in mice exposed to PFOS *in vivo*, it was hypothesized that similar effects would be seen in *in vitro* human studies. In addition, because immunotoxicity of PFAAs has been suggested to be related to proliferator-activated receptor (PPAR)- α activation (Yang *et al.*, 2000, 2002a, 2002b), this study assessed PPAR α as a possible mechanism for decreased IL-2 production.

Materials and Methods

Chemicals, Antibodies, and Supplies

Perfluorooctane sulfonic acid potassium salt (stated purity >98%) was obtained from Fluka Chemical (via Sigma, St. Louis, MO, USA; CAS No. 2795-39-3). PFOA was obtained from

Sigma-Aldrich/Fluka (Steinheim, Switzerland). The PPAR α antagonist, GW6471, was purchased from Tocris Bioscience (Bristol, United Kingdom). Human IL-2 enzyme-linked immunosorbent assay (ELISA) sets, assay diluent, coating buffer (pH 9.5), wash concentrate, stop solution, and substrate reagents A and B were obtained from BD Biosciences (San Jose, CA, USA). Anti-human CD3 and Anti-human CD28 were purchased from BD Pharmingen (San Diego, CA, USA). Phytohemagglutinin (PHA-P) and phorbol 12-myristate 13-acetate for molecular biology, 99% (TLC)-(PMA) were purchased from Sigma (St. Louis). Phosphate-buffered saline (without Ca²⁺ and Mg⁺) and RPMI-1640 medium (with l-glutamine and sodium bicarbonate) were purchased from Cellgro (Mediatech, Herndon, VA, USA). Non-essential amino acids (10 mM 100 \times), sodium pyruvate (100 mM), and antibiotic/antimycotic (100 \times) were obtained from Invitrogen (Gibco brand; Carlsbad, CA, USA). Fetal bovine serum was purchased from Gemini Bio-Products (West Sacramento, CA, USA). N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), flat bottom 96-well plates, and other disposables were obtained from Fisher Scientific (Atlanta, GA, USA). The naïve CD4⁺ T cell isolation kit (human) and LS columns used for magnetic isolation in the whole blood assay were purchased from Miltenyi Biotec (San Diego, CA, USA).

Cells

Jurkat cells were received from the American Type Culture Collection (ATCC, Manassas, Virginia). For all experiments using the Jurkat human T-cell line, the cells were maintained using standard tissue culture protocols. Cells were cultured in 75-cm² tissue culture flasks in supplemented RPMI-1640 medium (RPMI, 10% FBS, 1% antibiotic/antimycotic) and incubated under a humidified atmosphere of 5% CO₂/95% air at 37 °C. Growth medium was changed every 2 days.

Dosing-Jurkat Cell Line

Jurkat cells were plated in triplicate per dose on 96-well plates at 1×10^5 cells per well and stimulated with the combination of 1 $\mu\text{g ml}^{-1}$ PHA and 1 $\mu\text{g ml}^{-1}$ PMA, the combination of 1 $\mu\text{g ml}^{-1}$ anti-CD3 and 1 $\mu\text{g ml}^{-1}$ anti-CD28, or 1 $\mu\text{g ml}^{-1}$ anti-CD3 after optimization experiments. Cells were dosed with 0, 0.05, 0.1, 0.5, 1, 5, 10, 50, 75, or 100 $\mu\text{g ml}^{-1}$ PFOS only or 0, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, or 10 $\mu\text{g ml}^{-1}$ of PFOA only. These doses were chosen based on both exposure levels seen in humans and dose levels used in animal experiments (DeWitt *et al.*, 2009). As PFOS and PFOA do not dissolve readily in medium, dimethyl sulfoxide (DMSO) was used as a vehicle during the experiments (0.05% DMSO) and this constituted the vehicle control for the experiments (0 $\mu\text{g ml}^{-1}$ PFOS and 0 $\mu\text{g ml}^{-1}$ PFOA). In addition to single-component dosing, cells were also dosed with a combination of PFOS plus PFOA (0, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, or 10 $\mu\text{g ml}^{-1}$ PFOA + PFOS) to determine possible interactions as these two PFAAs are typically found in the environment together. In order to explore the role of the PPAR α mechanism, cells were also dosed with a PPAR α antagonist, GW6471 (5 μmol). DMSO was used as a vehicle for GW6471 (0.05% DMSO). Cells were incubated for 18 h at 37 °C (Cattan *et al.*, 2000). The supernatant from each triplicate was pooled by dose for each treatment, aliquoted, and stored at -80 °C until IL-2 analysis was performed using an IL-2 ELISA kit. Each experiment was repeated on three different days.

Human Blood Collection of CD4+ T Cells

Samples were collected from 11 healthy volunteer subjects under an approved Institutional Review Board (IRB) protocol. Criteria for exclusion were medication known to affect the immune system (i.e. steroids and non-steroidal anti-inflammatory drugs, or subjects suffering from an autoimmune disease). All subjects signed a consent form and were informed about methods and aims of the study. Six females and five males participated in the study. Blood samples (10 ml) were taken by venous puncture with heparin as the anticoagulant. Heparin was chosen over EDTA since EDTA can interfere with cell activation (Brunialti *et al.*, 2002).

Dosing-Human CD4+ T Cells

Red blood cells were lysed, white blood cells were collected, and CD4+ T cells were isolated from the white blood cell layer using magnetic separation. Cells were resuspended in supplemented RPMI-1640 media (RPMI, 10% FBS, 1% antibiotic/antimycotic) at 1×10^5 cells per well, stimulated with $1 \mu\text{g ml}^{-1}$ PHA and $1 \mu\text{g ml}^{-1}$ PMA, dosed with five different doses of PFOS: 0, 0.1, 1, 10, or $100 \mu\text{g ml}^{-1}$, and plated in triplicate per dose per individual on a 96-well plate. DMSO was used as a vehicle during the experiments (0.05% DMSO). Cells were incubated for 18 h at 37 °C. The supernatant from each triplicate was then pooled for each treatment by each individual, aliquoted, and stored at $-80 \text{ }^\circ\text{C}$ until IL-2 analysis was performed using an IL-2 ELISA kit.

Cell Viability

Cells were dosed as described above, incubated, and viability was assessed with Trypan blue dye via a hemocytometer (Strober, 2001). Living cells excluded the dye while dead cells were stained blue owing to the damaged cell membrane. Five squares were counted on the hemocytometer and viability was expressed as the percentage of living cells per total number of cells counted.

Statistical Analysis

All experiments with Jurkat cells were repeated at least three times, with representative results shown. Data are expressed as mean \pm standard error of the mean (SEM). Data were tested for normality (Shapiro–Wilk’s W-test) and homogeneity (Bartlett’s test for unequal variances). If need be, transformations were made. Statistical significance was determined using a one-way ANOVA ($P < 0.05$). Dunnett’s comparison was used to compare treatment groups to controls. Statistical analysis was performed using JMP version 10 (SAS Institute Inc., Cary, NC, USA).

Results

Effects of PFOS and PFOA on the Human Jurkat T Cell Line

Jurkat cells stimulated with PHA + PMA exhibited decreased IL-2 production after exposure to 50, 75, and $100 \mu\text{g PFOS ml}^{-1}$ (38%, 50%, and 61% decrease compared with the control, respectively, Fig. 1). PFOA exposure in cells stimulated with PHA/PMA resulted in no significant differences as compared with the control (Fig. 2). PFOS + PFOA exposure in

Jurkat cells also resulted in no significant differences in IL-2 production up to 10 μg PFOS + 10 μg PFOA ml^{-1} (data not shown). Addition of the PPAR α antagonist GW6471 to PFOS dosed cells stimulated with PHA + PMA resulted in decreased IL-2 production after exposure to 50, 75, and 100 μg PFOS ml^{-1} + 5 μmol GW6471 (66%, 87%, and 94% decrease compared with the control, respectively, Fig. 1). Addition of the PPAR α antagonist to PFOA-dosed cells stimulated with PHA + PMA resulted in no significant differences up to 10 μg PFOA ml^{-1} + 5 μmol GW6471 (Fig. 2). Jurkat cells stimulated with anti-CD3 + anti-CD28 showed no significant changes in IL-2 production after exposure to PFOS, PFOA, PFOS + PFOA, PFOS + GW6471, and PFOA + GW6471 (data not shown). Jurkat cells stimulated with anti-CD3 only showed decreased IL-2 production at 5, 10, 50, and 100 μg PFOS ml^{-1} (64%, 80%, 63%, and 96% decrease as compared with the control, respectively, Fig. 3). Exposure to PFOA, PFOS + PFOA, and PFOA + GW6471 with stimulation by anti-CD3 only was not examined as no significant differences were seen in PHA + PMA or anti-CD3 + anti-CD28 stimulation in Jurkat cells. Jurkat cell viability was not significantly different from the control at any of the *in vitro* exposure concentrations for PFOS (highest dose group exhibited 97% viability) or PFOA (highest dose group exhibited 100% viability).

Effects of PFOS on Primary Human CD4+ T Cells

Primary human CD4+ T cells were isolated and exposed to PFOS to determine if similar results were seen as with the immortalized Jurkat cell line. Only PFOS was examined, as no significant differences were noted with PFOA exposure at any dose level with Jurkat cells. IL-2 production was significantly decreased at 100 μg PFOS ml^{-1} , the highest PFOS dose in the primary human CD4+ T cells (86% decrease as compared with the control, Fig. 4). Cell viability was not significantly different from the control at any of the *in vitro* exposure concentrations (highest dose group exhibited 94% viability).

Discussion

Perfluorinated compounds have been shown to be immunotoxic (DeWitt *et al.*, 2009, 2012). PFOS has been shown to effect cytokine secretion in multiple studies. Corsini *et al.* (2011) demonstrated suppressed lipopolysaccharide (LPS)-induced tumor necrosis factors (TNF)- α and IL-6 secretion in human peripheral blood leukocytes starting at 0.1 μg ml^{-1} PFOS. LPS-induced release of TNF- α and IL-8 was also significantly reduced starting at 1 μg PFOS ml^{-1} in the human promyelocytic cell line THP-1. In this same study, PHA-stimulated peripheral blood leukocytes were examined and the addition of PFOS at 0.1, 1, and 10 μg PFOS ml^{-1} significantly decreased IL-4, IL-10, and IFN- γ production. Dong *et al.* (2011) showed decreases in numbers of T-cells secreting IL-2 (ELISPOT) at 50 mg kg^{-1} TAD over 60 days and Zheng *et al.* (2011) showed decreased numbers of T-cells producing IL-2 after a 7-day exposure to 20 mg kg^{-1} day $^{-1}$ PFOS (both in male C57Bl/6 mice). Peden-Adams *et al.* (2011) showed a significant decrease in IL-2 production in female B6C3F1 mice stimulated with anti-CD3 at 0.1, 0.5, and 5 PFOS (mg kg^{-1} TAD). In the current study, a significant decrease in IL-2 production was observed at 50, 75, and 100 μg PFOS ml^{-1} in PHA + PMA-stimulated Jurkat cells and a significant decrease at 5, 10, 50, and 100 μg PFOS ml^{-1} in anti-CD3-stimulated Jurkat cells. No other PFOS studies assessed this and are

available for comparison, however, our studies are consistent with the studies of Zheng *et al.* (2011), Dong *et al.* (2011) and Peden-Adams *et al.* (2011) which all demonstrated that IL-2 production was significantly decreased after PFOS exposure.

In addition to the Jurkat cell line, IL-2 production after PHA/PMA combined stimulation was examined in primary human CD4⁺ T cells and a significant reduction in IL-2 secretion was seen at the highest dose, 100 µg PFOS ml⁻¹. Therefore, the lowest observed effect level (LOEL) in primary human cells was 100 µg PFOS ml⁻¹ and the no observed effect level (NOEL) was 10 µg PFOS ml⁻¹. This NOEL corresponds to the NOEL from the Jurkat cell line after PHA/PMA combined stimulation. The LOEL in the Jurkat cell line after PHA/PMA combined stimulation was 50 µg PFOS ml⁻¹. This concentration was, however, not assessed in the primary human CD4⁺ cells. As a result of CD4⁺ cell isolation per individual in the primary CD4⁺ cell study the number of PFOS concentrations assessed was reduced to accommodate for less available cells for exposure. It is probable that the true LOEL in the primary cells may be between 10 and 100 µg PFOS ml⁻¹. Future studies should determine this. The overall LOEL of the study was 5 µg PFOS ml⁻¹ in Jurkat cells after being stimulated with Anti-CD3 with a NOEL of 1 µg PFOS ml⁻¹.

Although averaged PFOS serum levels over a 9-year period (1999–2008) are reported at 20.6 ng ml⁻¹ (0.021 ppm), this number may be deceiving (Kato *et al.*, 2011). This study excluded children under 12 years even although a recent study showed perfluorinated compound levels increasing from birth to 12 years of age in spite of discontinued manufacturing of PFOS and decreased manufacturing of PFOA (Schecter *et al.*, 2012). In addition, the Kato *et al.* (2011) study did not take into account humans exposed to higher concentrations of PFOS either occupationally or through other sources. The highest reported geometric mean for PFOS in the Kato *et al.* (2011) study was 30.4 ng ml⁻¹ (0.03 ppm) between 1999–2000, but PFOS serum concentrations have been reported to range up to 12 ppm (Fromme *et al.*, 2009). The current study demonstrated decreased IL-2 production starting at 5 µg PFOS ml⁻¹ (5 ppm), which suggests a possible risk of decreased IL-2 production owing to PFOS exposure in human populations at the higher end of the exposure range (up to 12 ppm). A significant decrease in IL-2 production could be detrimental as IL-2 is required for generation and maintenance of Tregs, which are needed to provide life-long protection from autoimmune disease (Malek, 2003).

Moreover, PFOS binds strongly to bovine serum albumin and studies have shown that the concentrations of PFOS required to saturate albumin would be in excess of 50–100 mg/l (ppm) possibly because PFOS is not available to other sites of action until the pool of available binding sites on albumin are occupied (Jones *et al.*, 2003). This is consistent with our study where most of the significant differences in IL-2 production were seen at PFOS concentrations above 50 ppm. One issue with *in vitro* studies in this case is that adverse effects of PFOS may not be seen until these binding sites are saturated. PFOS has been shown to have a high binding capacity for serum albumin in *in vitro* studies (Zhang *et al.*, 2009) and a study by Wambaugh *et al.* (2013) indicates that in *in vitro* systems studying PFAAs binding to proteins and lipids in medium along with portioning to the well of the wall may result in differences in chemical concentration between the administered concentration and the concentration at the site of action the cell. In fact, Levitt and Liss

(1986) concluded that *in vitro* toxic effects of PFOA and nonadecafluoro-*n*-decanoic acid (NDFDA) are reduced when serum is added to cell cultures. Therefore, if effects are seen in *in vitro* systems containing serum, it is likely they are truly caused by lower concentrations of PFAAs than the final well concentration indicates potentially making this study even more environmentally relevant.

While PFOS and PFOA are almost always found together in human serum samples, much less research is available on the effects of PFOA on cytokine production. Corsini *et al.* (2011) found that PFOA significantly reduced LPS-induced release of TNF- α in peripheral blood leukocytes at 1 and 10 $\mu\text{g PFOA ml}^{-1}$ and at 10 and 100 $\mu\text{g PFOA ml}^{-1}$ in THP-1 cells. In THP-1 cells, IL-8 was also significantly reduced at 100 $\mu\text{g PFOA ml}^{-1}$. In the same study, PHA-induced IL-4 and IL-10 was significantly decreased at 10 $\mu\text{g PFOA ml}^{-1}$ in peripheral blood leukocytes (Corsini *et al.*, 2011). To our knowledge, no studies have assessed the effect of PFOA on IL-2 production. In the current study, PFOA did not significantly reduce IL-2 production up to 10 $\mu\text{g PFOA ml}^{-1}$. Human exposure to PFOA is typically 10-fold lower than PFOS in the general public (Olsen *et al.*, 2007). Corsini *et al.* (2011) exposed a variety of human cell types to PFOS and PFOA and found that in all cases PFOS was able to inhibit cytokine production more than PFOA. In addition, a 2012 study by Corsini *et al.* (2012) found that of the six different perfluorinated compounds they examined, PFOA was the least active in terms of effects on cytokines. At current human exposure levels of PFOA, the present data would suggest that alterations in cytokines, specifically in IL-2 production, might not be seen. However, this could be confounded with known PFOS levels or by probable differences in final well concentrations and concentrations in the cells (as noted above). Our current study indicates that there is not any chemical interaction evident between the two in relation to *in vitro* T-cell IL-2 production in the Jurkat cell line at the concentrations utilized.

One possible mechanism of the decreased IL-2 production observed may be through the activation of the nuclear receptor peroxisome proliferator-activated receptor (PPAR)- α . This ligand activated nuclear receptor is a regulator of immune function, particularly inflammation (Daynes and Jones, 2002). Previous studies have shown that PFOS and PFOA can activate PPAR- α in humans and mice (Sohlenius *et al.*, 1992; Shipley *et al.*, 2004; Vanden Heuvel *et al.*, 2006). However, this mechanism may apply more to PFOA than to PFOS. PFOS has been shown to be less effective than PFOA at activating PPAR- α and neither PFOS nor PFOA were shown to have a significant activating effect on PPAR- γ (Takacs and Abbott, 2007). Peden-Adams *et al.* (2012) also showed that at environmentally relevant concentrations, PFOS does not upregulate expression of PPAR- α , γ , or δ genes. Corsini *et al.* (2011) showed that the effects of PFOA were dependent upon PPAR- α activation where effects of PFOS were independent of PPAR- α activation. Our results for PFOS were consistent with both of these studies where PFOS-induced suppression of IL-2 production was PPAR- α -independent and, therefore, a significant decrease in IL-2 production remained. Additional immune effects of PFOS may exist that are independent of PPAR- α . This is consistent with a previous study, which suggested that a PPAR- α -independent mechanism might contribute to the PFOS-induced suppression of IgM responses (DeWitt *et al.*, 2012) and production of TNF- α and IL-8 (Corsini *et al.*, 2011).

Corsini *et al.* (2012) has recently shown that the inhibitory effect of PFAAs on *in vitro* cytokine production (IL-6, TNF- α , IL-10 and IFN- γ) by human leukocytes can occur independently of PPAR α , and involves inhibition of NF- κ B activation.

T cells may be activated by a number of different agents. This study examined IL-2 production through stimulation of Jurkat cells using soluble PHA + PMA, soluble anti-CD3 + anti-CD28, or soluble anti-CD3 only. Significant decreases in IL-2 production were seen at increasing PFOS concentrations after stimulation with either PHA + PMA or anti-CD3. These significant decreases were also seen with PHA + PMA stimulation even when the PPAR α antagonist was added. Interestingly, no significant differences were seen after exposure to PFOS, PFOA, PFOS + PFOA, PFOS + GW6471, and PFOA + GW6471 using stimulation with anti-CD3 + anti-CD28. Activation of the T cell receptor (TCR)-CD3 complex results in signal 1 for T-cells which includes modulation of Lck, Fyn, Zap70, PLC γ , and activation of ERK, JNK, NF- κ B, and NFAT pathways. PHA stimulation results in crosslinking of the TCR. PMA stimulates PKC θ which targets NF- κ B and AP-1 activation, but requires a combination of TCR and CD28 stimulation for effective activation of NF- κ B and AP-1. PKC θ also interacts with calcineurin leading to activation of JNK and NFAT. CD28 provides a co-stimulatory signal to TCR activation providing for augmentation of IL-2 production through additional NF- κ B activation, but CD28 also activates Lck. Stimulation with anti-CD3 + anti-CD28 then activates both signal 1 (CD3 and CD28 with modulation of Lck) and 2 (CD28). Thus, each of the stimulants acts in a varied manner on signal 1 and signal 2 in the T-cell with PHA + PMA and anti-CD3 + anti-CD28 providing action in multiple locations along the pathways as compared with anti-CD3 alone. These results then suggest that adding direct co-stimulation of CD28 or PKC θ attenuates the decrease in IL-2 production from TCR stimulation alone. Corsini *et al.* (2011) found that PFOS caused inhibition of LPS-induced I- κ B degradation and decreased NF- κ B binding to DNA, p65 phosphorylation, and p65/p50 nuclear translocation in the THP-1 cell line. However, the current data suggests activation of NF- κ B in the Jurkat T-cell line by PHA + PMA or anti-CD3 + anti-CD28 stimulation attenuates the deficit in IL-2 production observed with anti-CD3 stimulation to varying degrees. Whether this is due to overcoming an NF- κ B signaling pathway deficit with additional stimulation or is due to other alterations in T-cell signaling is not clear and requires further study. However, this trend has been noted in patients with head and neck squamous cell carcinoma. Patients who were not responsive to anti-CD3 therapy were then given anti-CD3 + anti-CD28 stimulation, which enhanced IL-2 production (Shibuya *et al.*, 2000). Stimulation with anti-CD3 + anti-CD28 reversed immune unresponsiveness and induced a type 1 cytokine response. Therefore, anti-CD3 + anti-CD28 stimulation could be moderating the immunotoxicity of PFOS.

In conclusion, this is, to our knowledge, the first study to assess the effects of PFOS and PFOA on IL-2 production in both the Jurkat human cell line and primary human CD4+ T cells. PFOA did not appear to have any effect on IL-2 production, even at the highest concentration used in the Jurkat cells. Anti-CD3 was most effective at stimulating the Jurkat T cell line and provided the most sensitive data. A significant decrease in IL-2 production was seen with PFOS in both a human cell line and primary human CD4+ T cells. This suppression was seen at dose levels within the higher end of reported human exposure

ranges (Fromme *et al.*, 2009). A decrease in T-cell IL-2 production is characteristic of autoimmune diseases such as systemic lupus erythematosus (SLE). Because PFOS serum concentrations in humans have been reported to range up to 12 ppm (Fromme *et al.*, 2009) and a recent study demonstrated childhood deficits in immune system functions connected to exposure of PFOS and PFOA (Grandjean *et al.* 2012), further studies utilizing cells from autoimmune patients who have varying blood levels of PFOS and PFOA are underway to investigate the role of PFOS and PFOA as environmental triggers of autoimmune disease.

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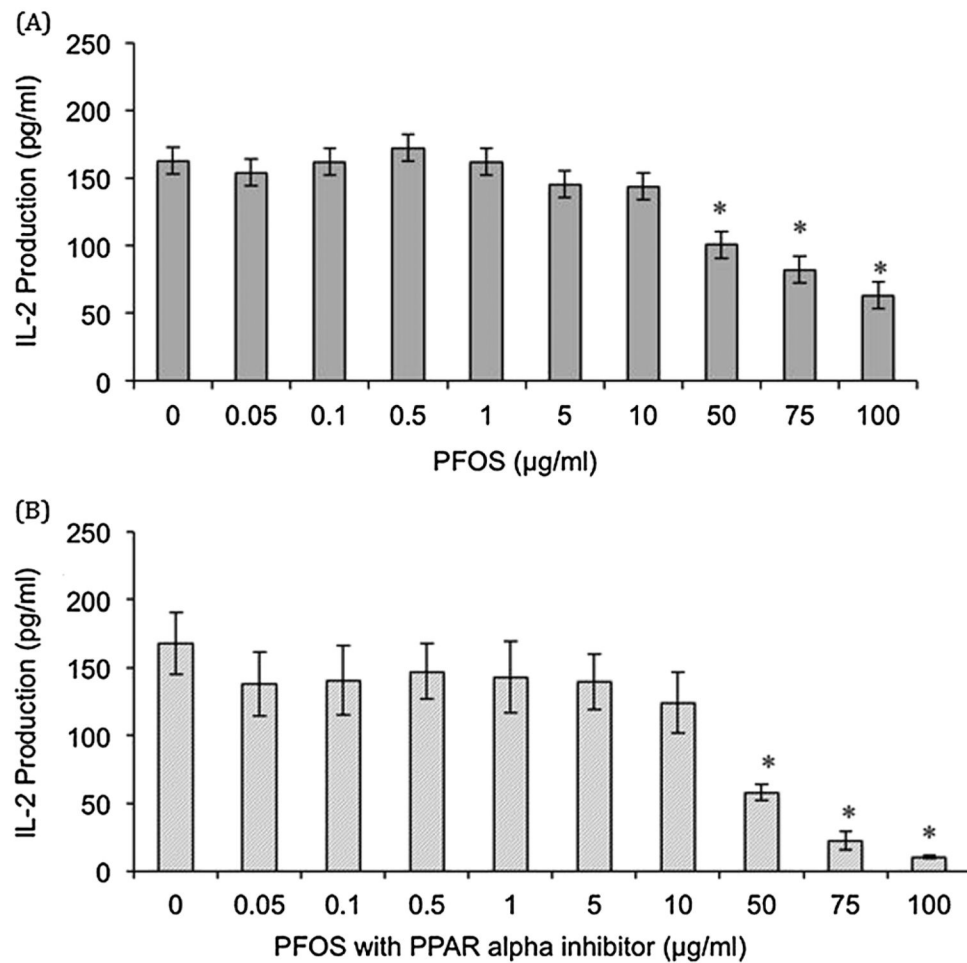


Figure 1.

In vitro effect of (A) perfluorooctane sulfonate (PFOS) and (B) PFOS plus a PPAR- α antagonist (GW6471) on interleukin (IL)-2 production in the Jurkat cell line stimulated with $1 \mu\text{g ml}^{-1}$ phytohemagglutinin (PHA) and $1 \mu\text{g ml}^{-1}$ phorbol myristate acetate (PMA). Each value represents the mean \pm standard error of the mean (SEM). The sample size for all treatments is six. *Significantly different from the control ($P < 0.05$). This experiment was conducted three times. Data from a single experiment are shown, as results are representative of experiments.

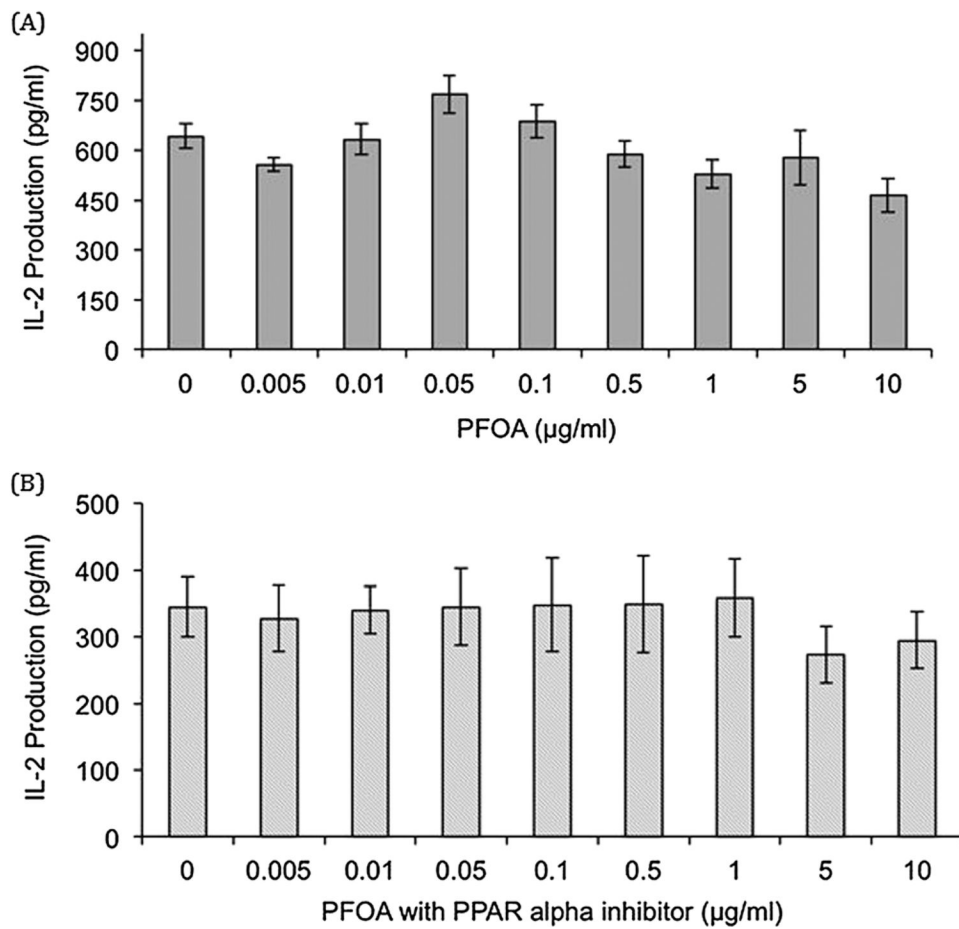


Figure 2.

In vitro effect of (A) perfluorooctanoic acid (PFOA) and (B) PFOA plus a PPAR- α antagonist (GW6471) on interleukin (IL)-2 production in the Jurkat cell line stimulated with $1 \mu\text{g ml}^{-1}$ phytohemagglutinin (PHA) and $1 \mu\text{g ml}^{-1}$ phorbol myristate acetate (PMA). Each value represents the mean \pm standard error of the mean (SEM). The sample size for all treatments is six. *Significantly different from the control ($P < 0.05$). This experiment was conducted three times. Data from a single experiment are shown, as results are representative of experiments.

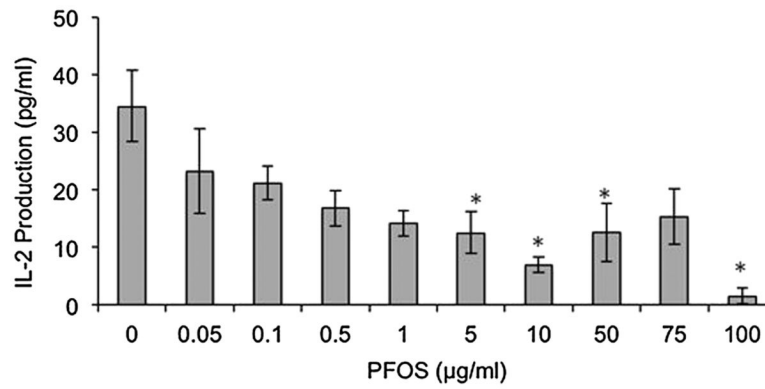


Figure 3.

In vitro effect of perfluorooctane sulfonate (PFOS) on interleukin (IL)-2 production in the Jurkat cell line stimulated with $1 \mu\text{g ml}^{-1}$ anti-CD3. Each value represents the mean \pm SEM. Sample size for all treatments is six. *Significantly different from control ($P < 0.05$). This experiment was conducted three times. Data from a single experiment are shown, as results are representative of experiments.

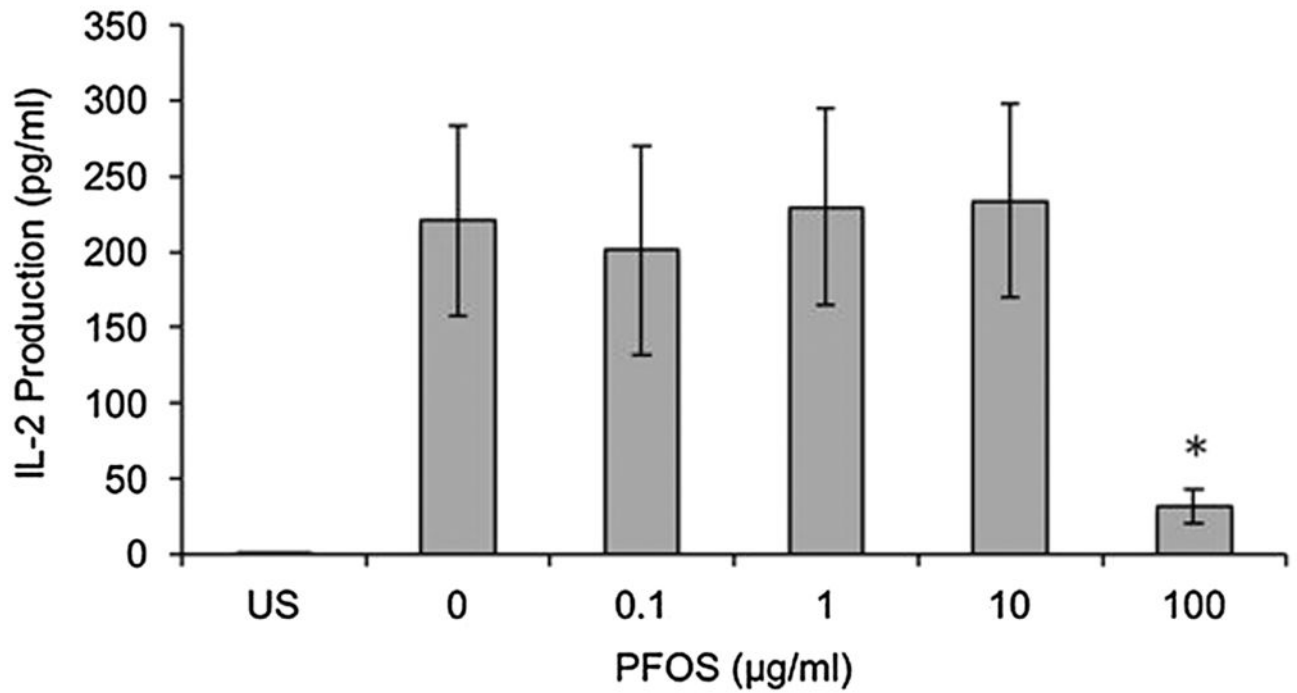


Figure 4.

In vitro effect of perfluorooctane sulfonate (PFOS) on interleukin (IL)-2 production in healthy human primary CD4⁺ T cells stimulated with 1 µg ml⁻¹ phytohemagglutinin (PHA) and 1 µg ml⁻¹ phorbol myristate acetate (PMA). Each value represents the mean ± SEM. The sample size for all treatments is 11 (6 females and 5 males). *Significantly different from the control ($P < 0.05$). US, unstimulated.