

# Acute Immunotoxic Effects of Perfluorononanoic Acid (PFNA) in C57BL/6 Mice

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## Abstract

Organic perfluorochemicals (PFCs) have become an environmental concern due to widespread detection in human blood and experimental evidence for immune, developmental, and liver toxicity. Whereas the blood concentrations of many PFCs are declining, blood levels of Perfluorononanoic Acid (PFNA) are rising in the United States. The purpose of the present studies was to determine the effects of PFNA on lymphoid organs and immune cells of C57BL/6 mice. The present study demonstrates that PFNA produces immunotoxic effects in both male and female C57BL/6 mice as evidenced by splenic atrophy, decreased splenocyte numbers, and a marked reduction in thymocyte viability. The current study also demonstrates that the effects of PFNA on different leukocyte populations are not uniform. The CD4<sup>+</sup>CD8<sup>+</sup> double-positive thymocytes were particularly sensitive to PFNA in which the proportion of this population was >95% decreased relative to the entire CD4<sup>+</sup> thymocyte population in PFNA-treated mice. Interestingly, PFNA also markedly increased serum levels of TNF $\alpha$  in response to LPS in mice. Collectively, the present studies demonstrate that PFNA decreases lymphocyte viability and alters the immune response to LPS in C57BL/6 mice.

**Keywords:** Perfluorononanoic acid; PFNA, Perfluoroalkyl acid; T cell; LPS; TNF $\alpha$

## Introduction

Organic perfluorochemicals (PFCs) are widely used in industry for a variety of applications, including food packaging, flame retardants, waterproof coatings, surfactants as well as in many other products [1-3]. Dozens of PFCs are used in manufacturing, among these are perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS), perfluorononanoic acid (PFNA) and the ammonium salts of these compounds [4]. PFCs have been manufactured since the 1950's, have low volatility, and are persistent in the environment [1,4]. The elimination half-lives of PFOA and PFOS in humans are estimated to be from 3 to 5 years [1,5]. Whereas the investigation of PFOA and PFOS has been a major area of toxicological research, other PFCs, such as PFNA, are not well characterized in this regard. Thus, in general, the sources, toxicokinetic and potential pathophysiological effects of PFNA in humans and animals necessitate further investigation.

Although most of the PFCs have not been extensively studied, evidence thus far suggests many of these compounds may cause immunological, developmental and liver toxicity [3,5,6]. With respect to PFNA, studies have demonstrated that prenatal exposure of mice to PFNA negatively impacts survival and development of pups [7]. PFNA administration also causes male-specific reproductive toxicity in rats, which is attributed to a defect in the secretory function of Sertoli cells [8,9]. In vitro studies indicate that PFNA causes only modest DNA damage at high concentrations, suggesting that genotoxicity is not a major concern [10]. Epidemiological data indicate a positive association between human serum levels of PFNA and total cholesterol, suggesting that PFNA exposure may alter cholesterol metabolism [11]. Of relevance to the present studies, daily administration of PFNA over 14 days causes apoptosis in spleens of BALB/c mice, an experimental mouse model of Th2-skewed immune responses [12].

The purpose of the present studies was to ascertain the effect of a single dose of PFNA (0.1 mmol/kg) on lymphoid organs and immune cell populations of C57BL/6 mice. The present studies are particularly important given that the immunotoxic effects of PFCs appear to be highly dependent upon mouse strain [6]. Whereas administration of 30 mg/kg/day of PFOA daily for 15 days causes a marked decrease in

weight of lymphoid organs in C57BL/6 mice, Sv/129 mice appear to be relatively insensitive to this effect. With respect to the current study, we are particularly interested in the C57BL/6 strain because it represents an experimental model that is not Th2-skewed and is also the most commonly-used mouse model in research.

## Materials and Methods

### Materials

PFNA (free acid form; 97% purity; M.W. 464 g/mol) was purchased from Sigma-Aldrich Co. (St Louis, MO).

### PFNA and LPS administration to mice

Eight-week-old adult male and female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, Maine), and housed according to AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) guidelines. Adult C57BL/6 male (n=5) and female (n=4) mice were administered a single i.p. dose of PFNA (0.1 mmol/kg of body weight), and control mice were treated i.p. with the vehicle, propylene glycol:water (1:1, v/v). After two weeks, the spleen, thymus, kidney, and liver were collected from each mouse. Lymph nodes were undetectable (or at least very difficult to distinguish) in PFNA-treated mice. For studies with LPS, LPS was administered two weeks after PFNA exposure. The mice were given a single i.p. dose of LPS or saline (1 mg/kg of body weight, diluted in saline). One hour and thirty minutes after LPS administration, blood was collected. The animal studies were conducted in accordance with the Guide for

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the Care and Use of Laboratory Animals as adopted by the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Kansas Medical Center and/or Michigan State University.

### Cell isolation

Spleens and thymuses were pulverized, made into single cell suspensions and filtered through nylon mesh. Leukocytes and red blood cells (RBCs) were counted using a hemacytometer (cells were counted in the presence and absence of a RBC-lysing agent).

### Flow cytometry

Freshly-isolated splenocytes were washed and resuspended in FACS buffer (PBS, 1% FCS). The cells were then incubated with anti-CD4/PerCP, anti-CD8/APC, anti-CD19/PE, and/or anti-CD14/FITC for 30 min at 4°C, after which the cells were washed and fixed with BD Cytofix fixation buffer (BD Biosciences, San Jose, CA). The fluorescence was then detected and quantified with a BD™ FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The data were analyzed using FlowJo 7.5 software (Tree Star, Inc., Ashland, OR).

### TNFα ELISA

TNFα levels in serum were quantified using a commercially available mouse TNF-α ELISA kit according to the manufacturer's protocol (Ebioscience, San Diego CA). The relative absorbance of each sample was quantified on a Bio-Tek μQuant microplate reader (Highland Park, VT). The TNF-α concentrations were calculated using a 4-parameter standard curve.

### Statistical analysis

The mean ± standard error was determined for each treatment in

the individual experiments. Homogeneous data were evaluated by two-way parametric analysis of variance. When significant differences were observed, the Holm-Sidak post-hoc test was used to compare treatment groups to the vehicle (VH) control using SigmaStat 3.01a software from Systat Software, Inc. (Chicago, IL).

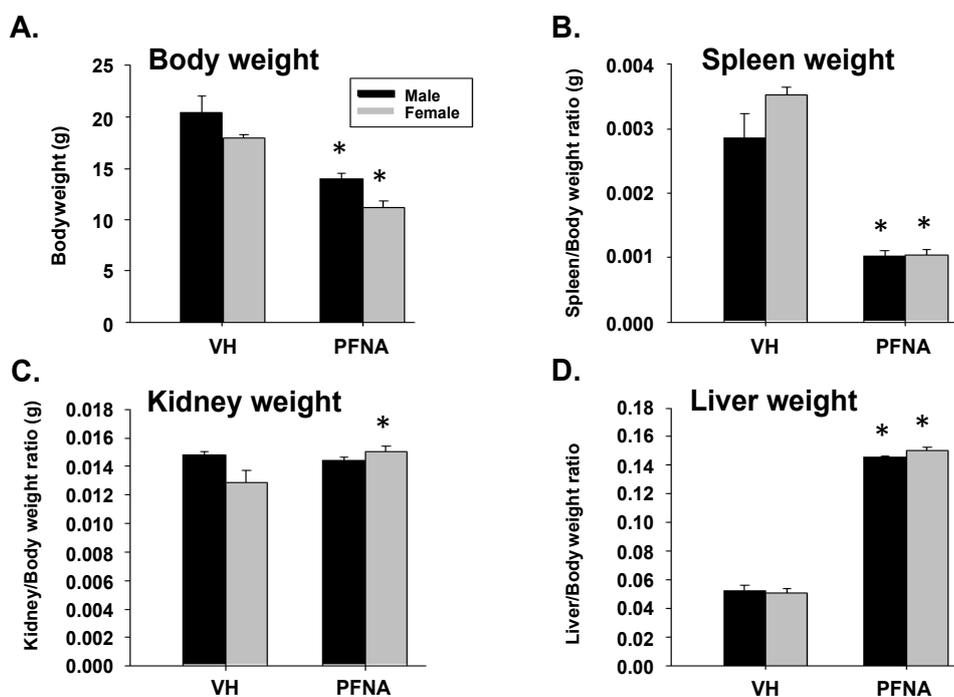
## Results

### Splenic atrophy by PFNA

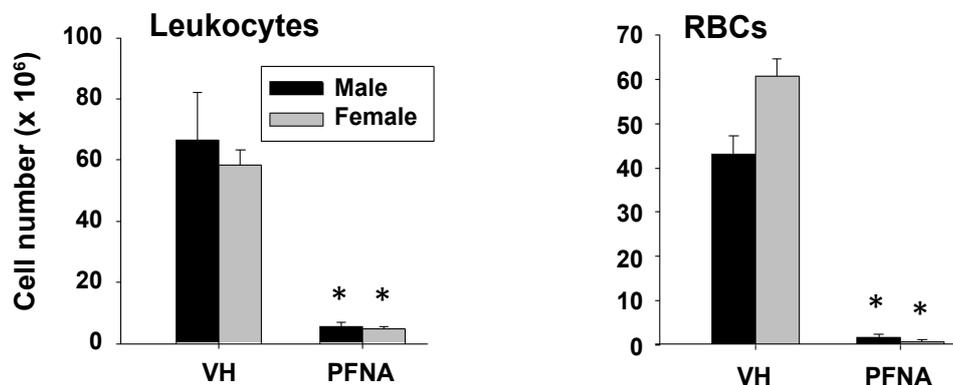
The immunotoxic effects of PFOA and PFOS have been described in previous studies [6]. To determine the acute effects of PFNA on the immune system, C57BL/6 mice were administered a single dose of PFNA (0.1 mmol/kg). Effects on lymphoid organs and immune cells were assessed two weeks later. PFNA caused a substantial decrease in body weight in both male (31%) and female (38%) mice (Figure 1A). Among the organs examined, the spleen was particularly sensitive to PFNA (with a 60-70% decrease in spleen to body weight ratio), whereas the decrease in kidney size was proportional to the decrease in overall body weight and thus no difference in the ratio of kidney to body weight was observed with PFNA treatment (Figure 1B and 1C). In contrast, PFNA administration resulted in increased liver size (≈300%, Figure 1D).

### PFNA decreases leukocyte viability

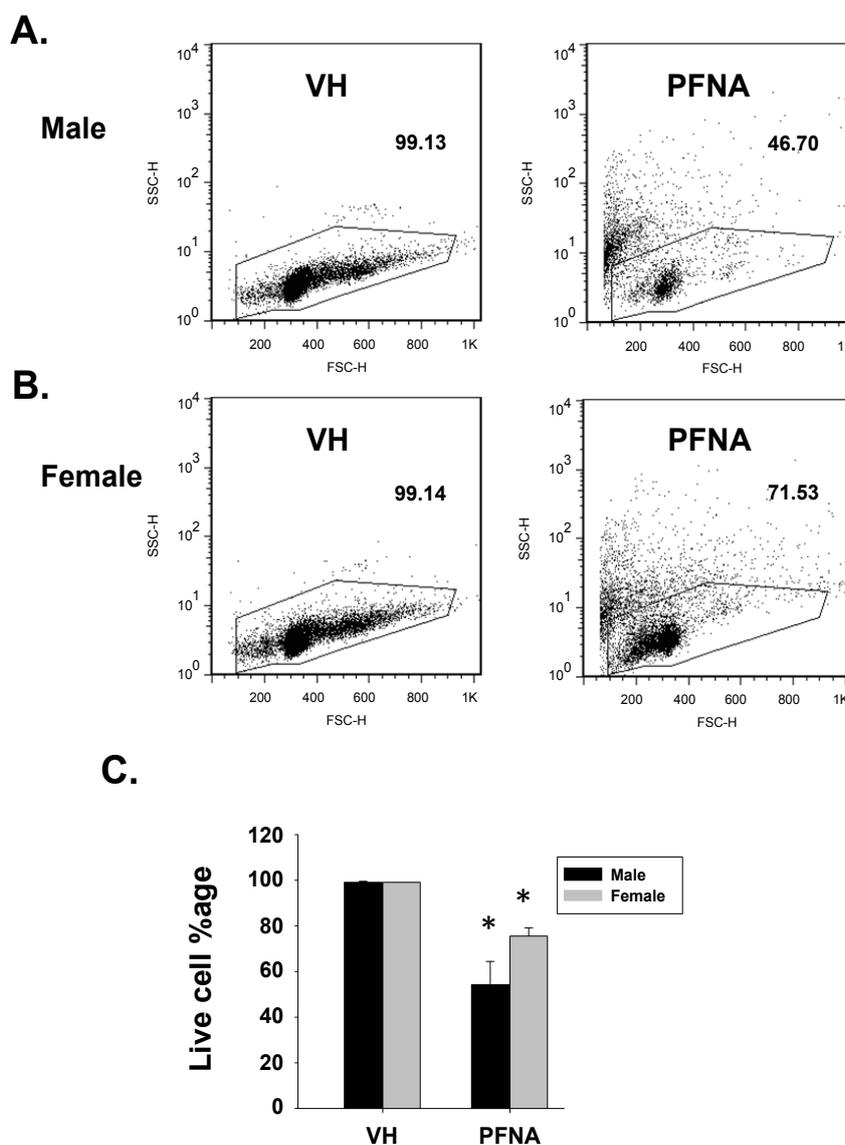
In correlation with the decrease in spleen size, a marked decrease in splenic leukocyte counts (~87.5% in males, and ~93% in females) and RBC counts (~95% in males, ~89% in females, Figure 2) was also observed. Altered forward vs. side scatter dot plots were observed in the thymocyte population from PFNA-treated mice (Figure 3). The decrease in forward scatter coupled with the increase in side scatter indicates the emergence of a population of apoptotic cells, which are smaller and denser than untreated lymphocytes.



**Figure 1:** Body and organ weights of mice two weeks after PFNA administration. (A) Body weight and the ratios of (B) spleen, (C) kidney, and (D) liver to body weight were measured two weeks after PFNA administration. The data are presented as the mean ± SE. \* denotes  $p < 0.05$  as compared to the vehicle (VH) control for the same gender.



**Figure 2:** PFNA markedly decreases leukocyte and RBC counts in spleen. Cells were diluted and counted in the presence and absence of an RBC-lysing agent. The data are presented as the mean  $\pm$  SE. \* denotes  $p < 0.05$  as compared to the vehicle (VH) control for the same gender.



**Figure 3:** PFNA decreases thymocyte viability as assessed by forward vs. side scatter. Forward vs. side scatter was assessed in (A) male and (B) female thymocytes by flow cytometry. (C) Live cells were quantified and graphed. The data are presented as the mean  $\pm$  SE. \* denotes  $p < 0.05$  as compared to the vehicle (VH) control for the same gender.

### Effect of PFNA on splenic T Cells

To determine the sensitivity of various leukocyte populations to PFNA, we conducted immunophenotyping analysis by flow cytometry. With respect to T cells, PFNA increased the ratios of both CD4<sup>+</sup> and CD8<sup>+</sup> cells relative to the entire splenocyte population in female mice (Figure 4). In male mice, PFNA increased the ratio of CD8<sup>+</sup> cells and caused a trend toward an increase of CD4<sup>+</sup> cells with respect to the entire splenocyte population. Overall, the data suggest T cells may be slightly less sensitive to the immunotoxic effects of PFNA compared to other splenocyte populations.

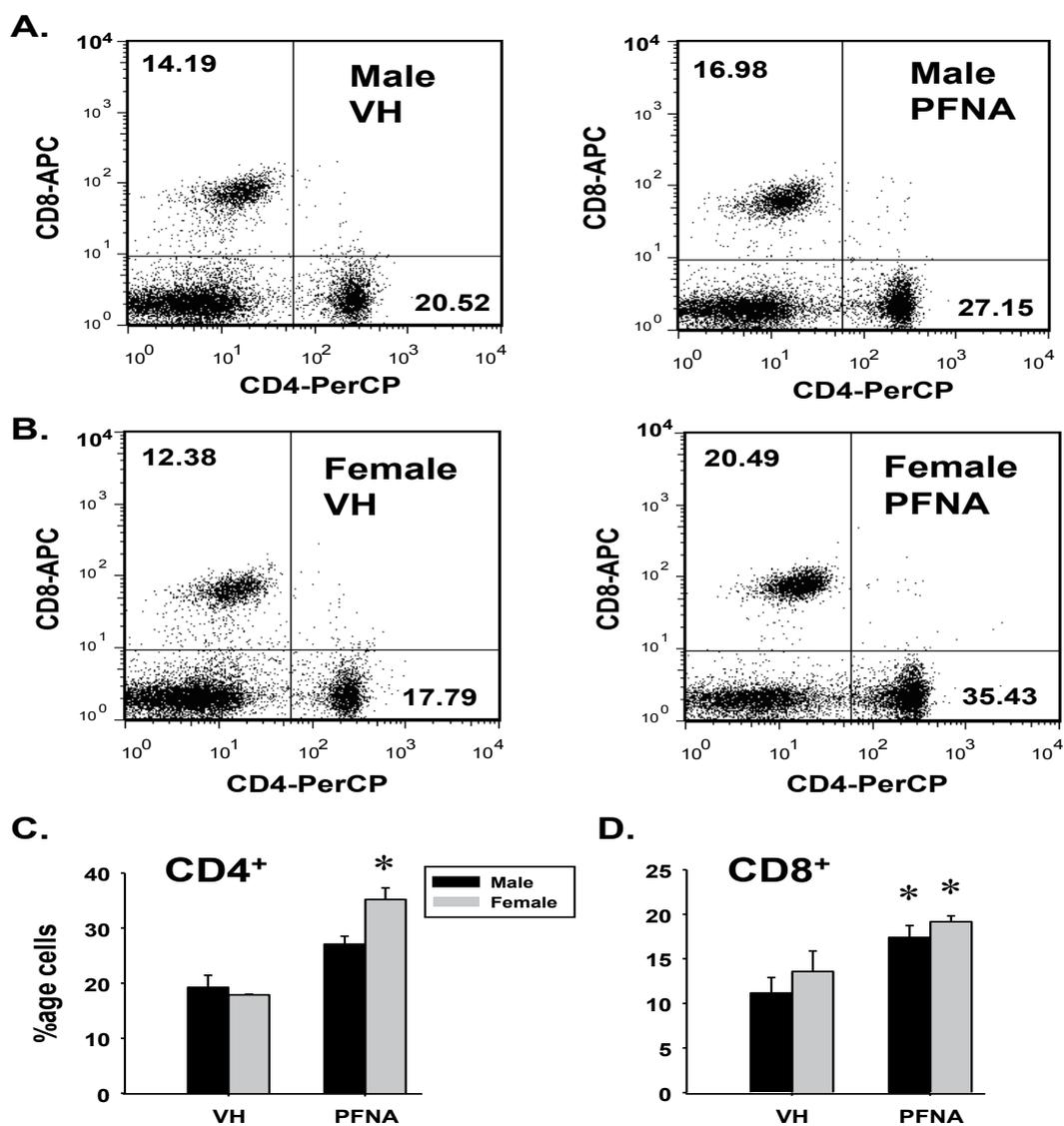
### PFNA alters immature T Cell populations

The effect of PFNA on immature T cells in the thymus was also determined. In addition to the marked decrease in viable cells in thymus

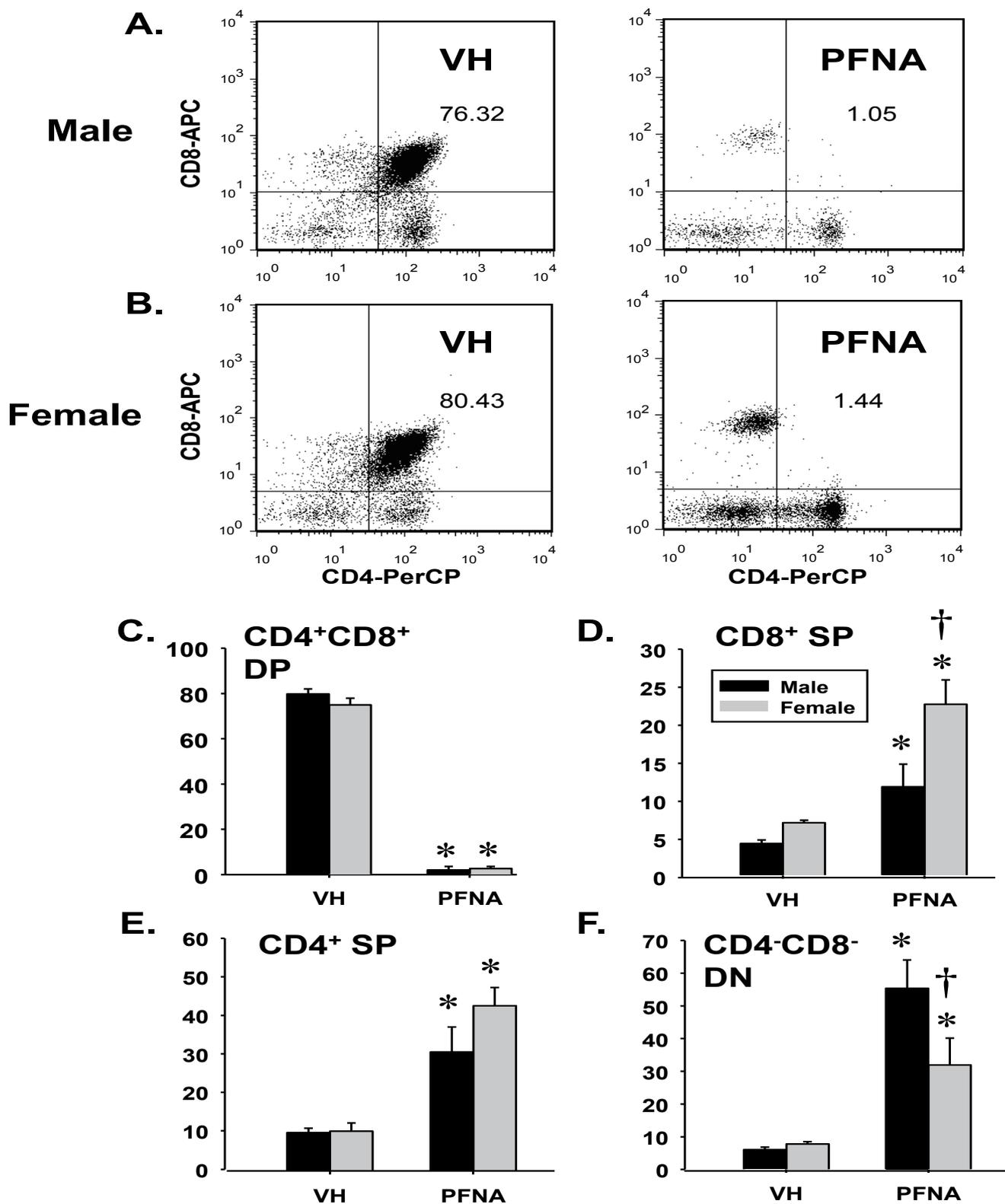
noted in Figure 3, substantial differences in thymic subpopulations were also observed (Figure 5 A and 5B). Notably, the CD4<sup>+</sup>CD8<sup>+</sup> double-positive population is largely absent in thymus from both male and female mice treated with PFNA (Figure 5C). With the decrease in the CD4<sup>+</sup>CD8<sup>+</sup> double-positive population, there was a concurrent increase in the ratios of the CD4<sup>+</sup> and CD8<sup>+</sup> single-positive populations as well as the CD4<sup>+</sup>CD8<sup>-</sup> double-negative population (Figure 5D - 5F). Collectively, the data suggest that although there is a decrease in overall thymocyte viability with PFNA treatment, the CD4<sup>+</sup>CD8<sup>+</sup> double-positive population is particularly sensitive to PFNA.

### Effects of PFNA on CD19<sup>+</sup> and CD14<sup>+</sup> Cells

In addition to CD4<sup>+</sup> and CD8<sup>+</sup> T cells, the effect of PFNA on CD19<sup>+</sup> and CD14<sup>+</sup> cells was also evaluated. CD19 is a coreceptor that associates with the B cell receptor and is thus, a commonly-used B cell marker.



**Figure 4:** PFNA increases the ratios of CD4<sup>+</sup> and CD8<sup>+</sup> T cells relative to the whole splenocyte population. Unfractionated splenocytes were washed, incubated with fluorochrome-conjugated antibodies and analyzed by flow cytometry. Representative dot plots of CD4<sup>+</sup> and CD8<sup>+</sup> T cells are depicted from VH-treated and PFNA-treated (A) male and (B) female mice. The numbers on the dot plot represent the percentage of the population for that quadrant. Graphical representation of (C) CD4<sup>+</sup> and (D) CD8<sup>+</sup> T cell percentage with respect to the whole splenocyte population. The data are presented as mean ± SE. \* denotes p<0.05 as compared to the vehicle (VH) control for the same gender.



**Figure 5:** Loss of the CD4<sup>+</sup>CD8<sup>+</sup> double-positive T cell population in thymus of PFNA-treated mice. Unfractionated thymocytes were washed, incubated with fluorochrome-conjugated antibodies and analyzed by flow cytometry. Representative dot plots of CD4<sup>+</sup> and CD8<sup>+</sup> T cells are depicted from VH-treated and PFNA-treated (A) male and (B) female mice. Graphical representation of (C) CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP), (D) CD8<sup>+</sup> single-positive (SP), (E) CD4<sup>+</sup> SP, (F) CD4-CD8<sup>-</sup> double-negative (DN) T cell percentages with respect to the whole splenocyte population. The data are presented as mean ± SE. \* denotes p<0.05 as compared to the vehicle (VH) control for the same gender. † denotes p<0.05 as compared between genders for the same treatment group.

CD14 is the LPS co-receptor and is used as a marker of phagocytes, such as macrophages and dendritic cells. The data demonstrate that PFNA decreases the ratio of CD19<sup>+</sup> cells within the splenocyte population in female mice (Figure 6). Although not statistically significant, PFNA tended to decrease the ratio of CD19<sup>+</sup> cells in male mice as well. In addition, PFNA decreased the ratio of CD14<sup>+</sup> cells within the splenocyte population in both male and female mice (Figure 7).

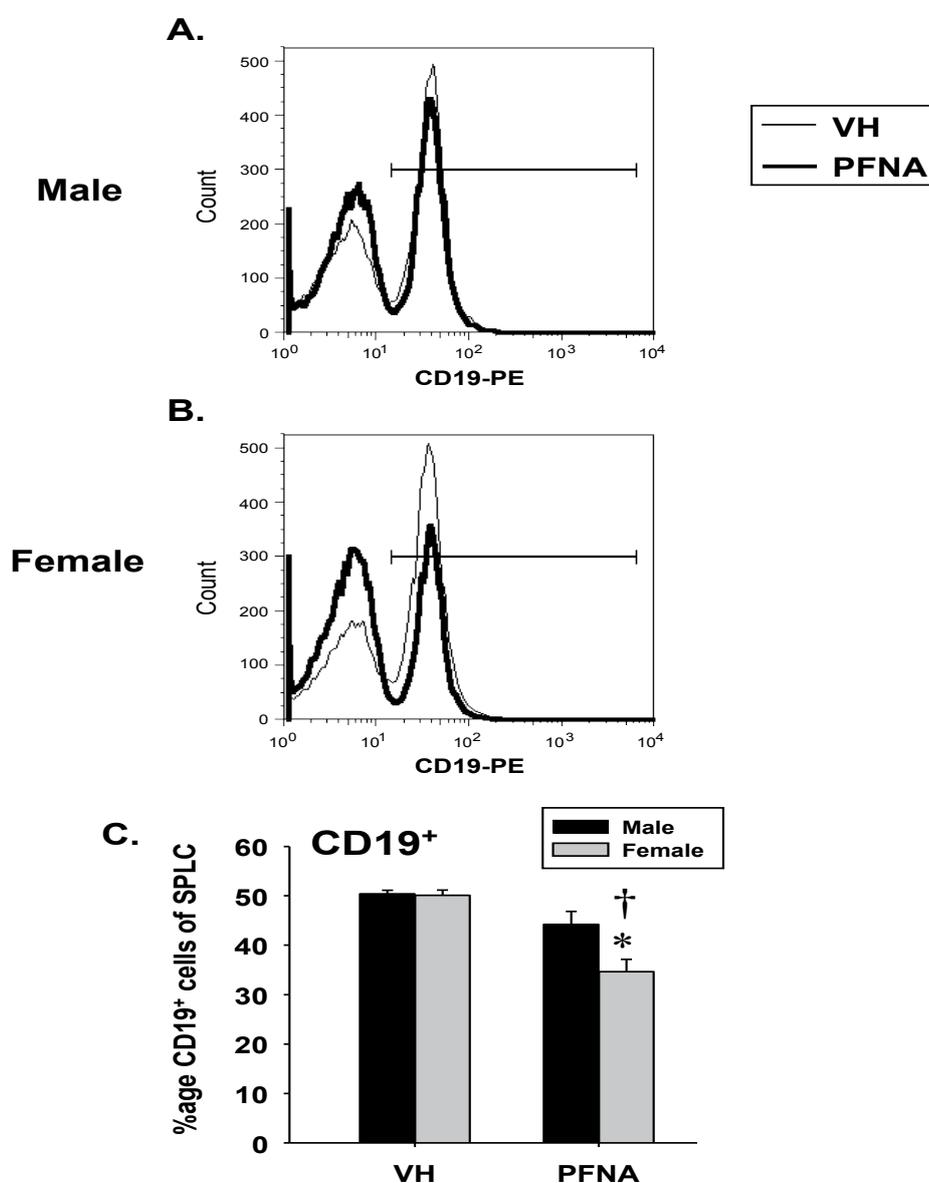
### PFNA increases TNF $\alpha$ in response to LPS

Because CD14<sup>+</sup> cells in spleen appeared to be particularly sensitive to PFNA as shown in Figure 7, we investigated the effect of PFNA on induction of TNF $\alpha$  in LPS-treated mice, a response mediated in large part by CD14<sup>+</sup> cells. Interestingly, PFNA administration caused a

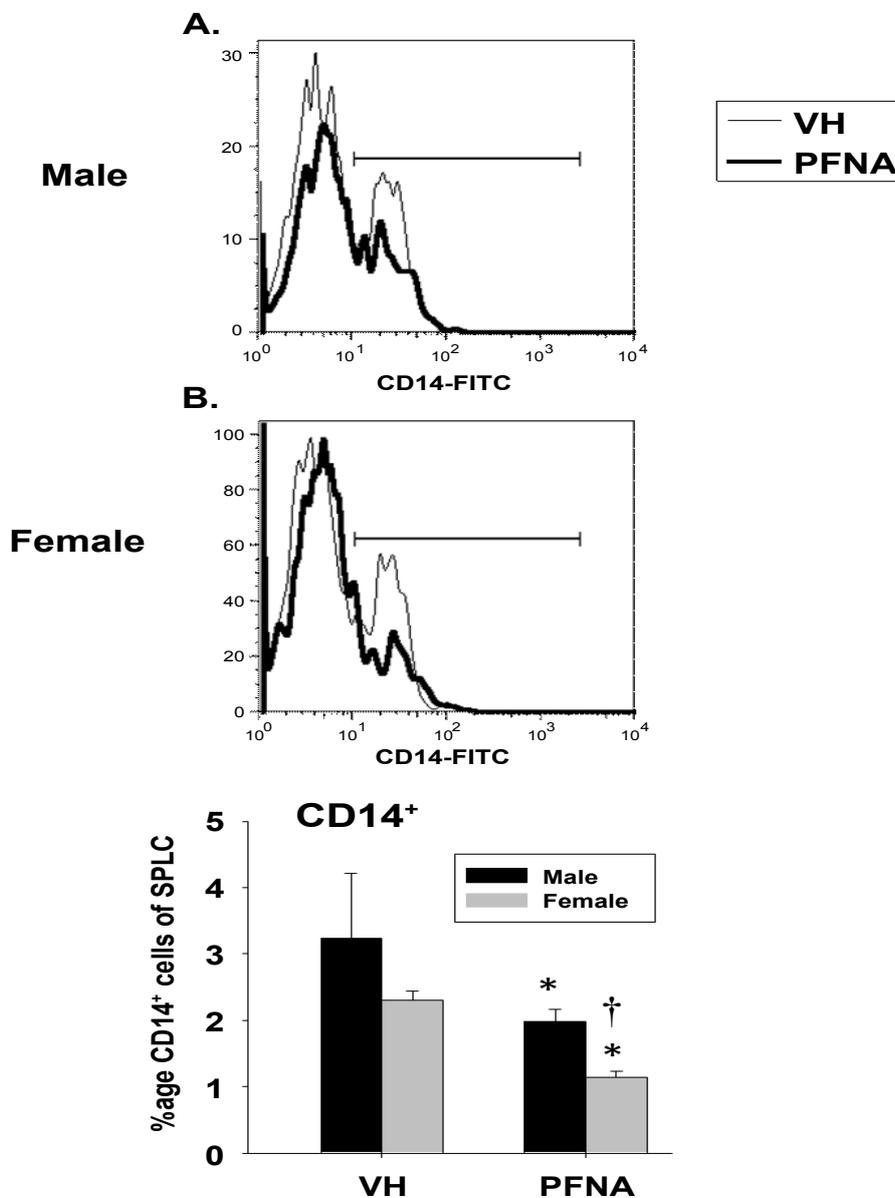
marked increase in TNF $\alpha$  production in LPS-treated mice, which was an unexpected finding (Figure 8).

### Discussion

The present studies are the first to demonstrate that acute exposure to PFNA results in severe immunotoxicity in both male and female C57BL/6 mice. Specifically, the current studies show that a single dose of PFNA (0.1 mmol/kg) substantially decreases spleen size in male and female mice, which correlates with markedly decreased numbers of RBCs and leukocytes in spleen. Furthermore, the sensitivity of various leukocyte populations to PFNA is not uniform. In thymus, PFNA toxicity is most evident in the CD4<sup>+</sup>CD8<sup>+</sup> double-positive population, which represents the most rapidly dividing population in thymus.



**Figure 6:** Decrease in the percentage of CD19<sup>+</sup> cells in spleen of female PFNA-treated mice. Unfractionated splenocytes were washed, incubated with fluorochrome-conjugated antibodies and analyzed by flow cytometry. A representative histogram of CD19<sup>+</sup> B cells is depicted from VH-treated (shown in thin gray line) and PFNA-treated (shown in heavy black line) (A) male and (B) female mice. (C) Graphical representation of CD19<sup>+</sup> B cell percentage with respect to the whole splenocyte population. The cells were gated on those that were CD4<sup>+</sup>CD8<sup>+</sup>. The data are presented as mean  $\pm$  SE. \* denotes p < 0.05 as compared to the vehicle (VH) control for the same gender. † denotes p < 0.05 as compared between genders for the same treatment group.



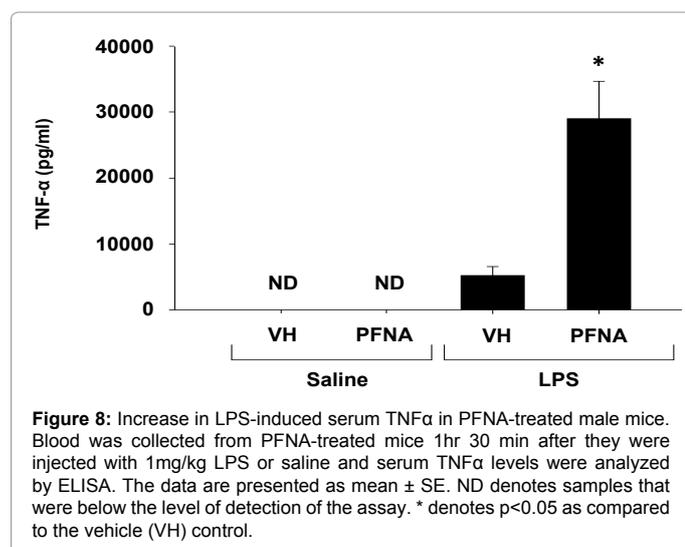
**Figure 7:** Decrease in the percentage of CD14<sup>+</sup> cells in spleen of male and female PFNA-treated mice. Unfractionated splenocytes were washed, incubated with fluorochrome-conjugated antibodies and analyzed by flow cytometry. The cells are gated on the CD4<sup>+</sup>CD8<sup>+</sup>CD19<sup>-</sup> population. A representative histogram of CD14<sup>+</sup> cells is depicted from VH-treated (shown in thin gray line) and PFNA-treated (shown in heavy black line) (A) male and (B) female mice. (C) Graphical representation of CD14<sup>+</sup> cell percentage with respect to the whole splenocyte population. The cells were gated on those that were CD4<sup>+</sup>CD8<sup>+</sup>CD19<sup>-</sup>. The data are presented as mean  $\pm$  SE. \* denotes p<0.05 as compared to the vehicle (VH) control for the same gender. † denotes p<0.05 as compared between genders for the same treatment group.

Interestingly, a marked increase in TNF $\alpha$  production in response to LPS was observed in PFNA-treated mice compared to vehicle controls. Overall, the current studies demonstrate decreased leukocyte viability in spleen and thymus as well as enhanced response to LPS by a single dose of PFNA in C57BL/6 mice.

The pronounced decrease in lymphoid organ weight and leukocyte numbers by PFNA is strikingly similar to the effects of other PFCs, such as PFOA. Like PFNA, PFOA administration causes considerable splenic atrophy and a concomitant decrease in the number of splenocytes [13]. In addition, the CD4<sup>+</sup>CD8<sup>+</sup> double-positive population in thymus is more sensitive to PFOA than other thymic populations, which is consistent with the effects of PFNA (Figure 5). PFOA also diminishes

immune function as assessed by IgM production in the plaque-forming colony assay [14,15]. In contrast to PFNA and PFOA, PFOS does not decrease spleen and thymus weights in Sprague-Dawley rats or B6C3F1 mice [16,17]. The current studies in male and female C57BL/6 mice are consistent with previous observations of toxicity in lymphoid organs by PFNA in male Sprague-Dawley rats and male BALB/c mice [12,18,19]. Collectively, the present studies indicate that PFNA-mediated immunotoxicity resembles that of other PFCs, in particular, PFOA.

Previous studies indicate that a number of PFCs, including PFOA and PFOS, cause peroxisome proliferation in hepatocytes, which is associated with activation of peroxisome proliferator-activated receptor



alpha (PPAR $\alpha$ ) [20-22]. Of importance to the current studies, PPAR $\alpha$  agonists have been shown to have a number of immunomodulatory effects [6]. Administration of the synthetic PPAR $\alpha$  agonist, Wy-14,643, decreased spleen weight and splenic cellularity as well as thymus weight and thymic cellularity in C57BL/6 mice [23]. Furthermore, PFOA exposure decreased spleen weight and splenic cellularity, in wild-type, but not PPAR $\alpha$ -null mice. The decreased thymus weight and cellularity by PFOA was attenuated, but not eliminated, in PPAR $\alpha$ -null mice, suggesting the effects in thymus are partially mediated through PPAR $\alpha$ . The role of PPAR $\alpha$  in PFOA-mediated toxicity remains controversial, however, because hepatomegaly by PFOA is observed in PPAR $\alpha$ -null mice, suggesting this effect is independent of PPAR $\alpha$  [23]. Further studies are needed to determine the role of PPAR $\alpha$  in PFNA-mediated immunotoxicity.

The enhanced TNF $\alpha$  response to LPS in PFNA-treated mice was an unexpected finding. Because there was significant splenic and thymic atrophy and decreased leukocyte viability in PFNA-treated mice, we initially hypothesized that PFNA would decrease the TNF $\alpha$  response to LPS. The marked increase in TNF $\alpha$  production in response to LPS by PFNA suggests that PFNA treatment primes CD14<sup>+</sup> phagocytes for an inflammatory response. This may be due to the marked decrease in leukocyte viability in PFNA-treated animals. Cell death, in particular necrosis, triggers inflammatory response or priming of macrophages and other phagocytes and thus could cause an enhanced TNF $\alpha$  response to LPS.

The average serum concentration of PFNA in the U.S. population is approximately 1.5 ng/ml [24,25]. However, it is important to note that there is significant interindividual variability in PFNA serum levels, ranging from 5 to 50 times higher than the mean, due to numerous factors, such as age, race and gender, among other factors [24,26]. It has also been reported that populations living near PFC manufacturing facilities have significantly higher PFC blood concentrations than the national mean in the U.S., which reach as high as 300 times greater than average in certain communities [27]. Occupational exposure of workers within the manufacturing facility is potentially even higher. Although an epidemiological study on the effects of occupational PFNA exposure on blood lipids and liver enzyme activity has been published, the blood concentrations of PFNA in exposed workers was not reported in this study [28]. Given that PFNA blood concentrations have recently doubled over the course of 6 years in the U.S., all of these factors are of

potential concern [29]. Indeed, given that PFNA blood concentrations in other industrialized countries are reported to be higher than in the U.S., PFNA is a matter of global concern [30]. Our focus has been on the effects of a single high dose exposure to PFNA, which is potentially relevant to inadvertent high dose occupational exposure as well as to sensitive populations in which PFNA blood concentrations are higher than the mean or a combination of both of these factors.

Collectively, the findings of the present studies demonstrate severe immunotoxic effects from a single dose of PFNA, including marked lymphoid organ atrophy, splenic and thymic cellularity, and altered ratios of leukocyte populations. Further investigation will be needed in order to better predict the consequences of acute PFNA exposure on immune organs and immune cellularity in humans.

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