In vivo immunotoxicity of perfluorooctane sulfonate in BALB/c mice: Identification of T-cell receptor and calcium-mediated signaling pathway disruption through gene expression profiling of the spleen

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ABSTRACT

Perfluorooctane sulfonate (PFOS) is a persistent organic pollutant that is used worldwide and is continuously being detected in biota and the environment, thus presenting potential threats to the ecosystem and human health. Although PFOS is highly immunotoxic, its underlying molecular mechanisms remain largely unknown. The present study examined PFOS-induced immunotoxicity in the mouse spleen and explored its underlying mechanisms by gene expression profiling. Oral exposure of male BALB/c mice for three weeks followed by one-week recovery showed that a 10 mg/kg/day PFOS exposure damaged the splenic architecture, inhibited T-cell proliferation in response to mitogen, and increased the percentages of T helper (CD3⁺CD4⁺) and cytotoxic T (CD3⁺CD8⁺) cells, despite the decrease in the absolute number of these cells. A delayed type of PFOS immunotoxicity was observed, which mainly occurred during the recovery period. Global gene expression profiling of mouse spleens and QRT-PCR analyses suggest that PFOS inhibited the expression of genes involved in cell cycle regulation and NRF2-mediated oxidative stress response, and upregulated those in TCR signaling, calcium signaling, and p38/MAPK signaling pathways. Western blot analysis confirmed that the expressions of CAMK4, THEMIS, and CD3G, which were involved in the upregulated pathways, were induced upon PFOS exposure. Acute PFOS exposure modulated calcium homeostasis in splenocytes. These results indicate that PFOS exposure can activate TCR signaling and calcium ion influx, which provides a clue for the potential mechanism of PFOS immunotoxicity. The altered signaling pathways by PFOS treatment as revealed in the present study might facilitate in better understanding PFOS immunotoxicity and explain the association between immune disease and PFOS exposure.

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1. Introduction

Perfluoroalkyl acids (PFAAs) are a family of synthetic chemicals that have been widely used in industrial and commercial products such as surfactants, lubricants, fabric and paper coatings, fire-fighting foams, and stain repellants [1]. Among these compounds, perfluorooctane sulfonate (PFOS) is the most studied member due

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to its extensive distribution [2], long half-life of elimination (about 5.4 years in humans) [3], and bioaccumulation. As a persistent organic pollutant, PFOS has been continually detected in various environmental and biological samples, including humans [2,4,5], even after its voluntary phase-out, probably due to its stability and the biotransformation of its precursor compounds (such as fluorotelomer alcohols) that are still produced in large quantities [6]. Humans are exposed to PFAAs mainly through oral consumption and inhalation [7], which include dietary intake [8], dirty air, and indoor dust ingestion [9], as well as lactational transfer [10]. The serum PFOS concentration of occupational exposure workers ranges from 145 to 3490 ng/mL [3]; the highest PFOS reported in blood is 12,830 ng/mL [11] and in the general population, the serum PFOS concentration was found to be within the range of 0.5–92.9 ng/mL [12]. Epidemiological data showed that PFAA

(including PFOS) exposure was associated with various adverse health effects, including immunodeficiency such as lower antibody responses to childhood immunizations [13] and juvenile asthma [14]. The contamination of PFOS presents potential threats to the ecosystem and human health, thus raising great concerns.

Lines of evidence obtained from animal models strongly suggest that PFOS is immunotoxic [15]. Oral PFOS exposure induces lymphoid organ atrophy, alterations in lymphoid cellular composition, and changes in *de novo* antibody synthesis, which in turn could lead to a decrease in host resistance to influenza A [16–18]. PFOS exposure has also been shown to decrease the number of T-cell subpopulations and depress the natural killer cell activity of cells in the spleen of adult male C57BL/6 mice [19,20]. PFOS also causes apoptosis in splenocytes and thymocytes [21,22], and interfere with the release of pro-inflammatory cytokines such as TNF- α , interleukin (IL)-1 β , IL-2, IL-4, and IL-6, after short-term or subchronic PFOS exposure [22–24].

The reported side effects of PFOS on the immune system has resulted in a few studies that addressed alterations of signaling transduction in immune cells, although the underlying molecular mechanism of these effects remain unclear. Previous mechanistic studies using non-immune cell models might provide invaluable clues on the mechanisms of PFOS immunotoxicity. For example, the studies based on neuron and liver models indicated that PFOS could disrupt the peroxisome proliferator receptor (PPAR) α -mediated pathway, resulting in disruption of lipid metabolism, inflammation, and inhibition of energy homeostasis and cell differentiation [25–27]. Some other studies indicated that PPAR α -independent pathways might also be involved in PFOS toxicity because the toxic effects still occurred even in PPAR α -null models [23,28,29]. Alternatively, these alterations might merely pose an indirect stress effect on immune cells [15].

In the immune system, the spleen plays an important role in initiating immune responses to blood-borne antigens through humoral and cell-mediated pathways [30]. Its dysfunction is usually associated with diminished responsiveness to antigens and increased susceptibility to infection [31]. The bioaccumulation of PFOS in the spleen has been previously reported in rodents [17,32], and several studies have shown that PFOS-induced immunologic outcomes in the spleen involve reduction in weight, structural damages, change in cellular composition, and inflammation [19,33]. To date, information on the underlying mechanisms of these effects is limited. Therefore, in the present study, we hypothesized that PFOS exposure causes damage to the spleen and interferes with multiple pathways that are related to immune cell signaling transduction. Due to the complexity of PFOS toxicity, multiple mechanisms might be involved, but pathways are usually intermingled and crosstalk between each other. The microarray technique has the advantages of taking a snapshot of the expression of thousands of genes in parallel and generating a large amount of data that can potentially provide systems-level information regarding the underlying mechanisms [34]. To test the hypothesis, we orally exposed BALB/c mice to PFOS at levels ranging from 0 to 210 mg/kg total administered dose (TAD), which was comparable to that utilized by Wang et al. [33] and Zheng et al. [19] (0–280 mg/kg TAD), for a period of three weeks, which was followed by one week of recovery. The one week recovery was set to observe the reversibility, retardance or incubation period for PFOS-induced immunotoxic effects, as PFOS was shown to bio-accumulate/persist in living bodies. The toxicological effects of PFOS on the spleen were first measured, and then the microarray technique was employed for gene expression profiling of the mouse spleen to explore its underlying mechanism.

2. Materials and methods

2.1. Chemicals

Perfluorooctane sulfonic acid potassium salt (PFOS) (CAS number, 2795-39-3; purity > 98%) and concanavalin A (Con A) was purchased from Sigma–Aldrich (St. Louis, MO, USA). PFOS was prepared in double-distilled H₂O with 0.5% Tween-20 [33]. Con A was dissolved in DMSO.

2.2. Animals

Male BALB/c mice (4–5 weeks of age) were purchased from the Department of Laboratory Animal Science, Peking University (Beijing, China) and bred and housed under pathogen-free conditions at the animal care facility. All of the animal experiments conformed to the principles of care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Peking University. The mice were randomly distributed into four groups, with each group consisting of 28 mice. The four groups of mice were orally exposed to 0, 2.5, 5, and 10 mg/kg/day PFOS, respectively, via gastric gavage, and the vehicle control group (0 mg/kg/day) was treated with an equal volume of ddH₂O (with 0.5% Tween-20). The treatment continued for 3 weeks, followed by a one-week recovery period. The mice in each group were sacrificed by cervical dislocation every 7 days and the spleens were immediately isolated, weighed, and divided into three portions for the following treatments: one portion was fixed in 4% para-formaldehyde for histological examination (see in Supplementary materials.), the second portion was frozen and stored at –80 °C for RNA and protein extraction, and the third was immediately made into a single-cell suspension and red blood cells were lysed for lymphocyte phenotyping and lymphoproliferation analyses. Additionally, a hemocytometer was used to count the number of splenocytes in cell suspension and Trypan blue exclusion was used to assess cell viability, which was always >90%.

2.3. Lymphocyte phenotyping

The T lymphocyte composition of the spleens was analyzed on a BD LSRII flow cytometer (BD Biosciences, San Jose, CA, USA) by using a Mouse T Lymphocyte Subset Antibody Cocktail (BD Pharmingen, San Diego, CA, USA). The single-cell suspension of the splenocytes (1×10^7 nucleated cells/mL PBS) was incubated with antibodies for 30 min at 4 °C in the dark. The specific details of this experiment are presented in the Supplementary materials. Isotypic antibody controls were used to set up gates for the T lymphocyte subset (CD3⁺, CD3⁺/CD4⁺, and CD8⁺) phenotyping. The typical gate setting and quadrant analyses are shown in [Supplementary Fig. S1](#). Data were represented as the percentage of the total number of cells. The absolute number of particular cell population was measured and expressed as the ratio of cell number in treatment group after normalization against that in control group.

2.4. Lymphoproliferation assay

The single-cell suspension was adjusted to a density of 2×10^6 nucleated cells/mL in RPMI-1640 medium containing 10% heat-deactivated FBS (cRPMI-1640) and dispensed into a 96-well plate (100 μ L/well) containing triplicate wells of either 5 μ g/mL Con A or cRPMI-1640 (as unstimulated wells). The final volume of each well was 200 μ L. The plates were incubated for 72 h at 37 °C in a humidified 5% CO₂–air mixture. After incubation, 10 μ L MTT was

added into each well, the plate was incubated for 4 h, followed by the addition of 100 μ L of DMSO to dissolve the formazan crystals. The microplate was then read on a microplate reader (Thermo-Varioskan, MA, USA) at a wavelength of 490 nm.

2.5. Total RNA isolation and microarray analysis

The spleens of mice in the 10 mg/kg/day group at week 3 were used for microarray analysis. Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). The Agilent 2100 Bioanalyzer System (Agilent Technologies, Inc., Waldbronn, Germany) was used to assess the quality and quantity of purified total RNA. Only the samples that passed the RNA quality test were used in the subsequent assays. The GeneChip® Mouse Genome 430 2.0 Array (Affymetrix Inc., Santa Clara, CA, USA) was used, and the experiments were performed according to standard procedures. More details can be found in the Supplementary Materials. Three biologic replicates were independently performed for both treatment and control groups (i.e., three sets of microarray data were separately collected from three spleens in each group). Data are deposited at Gene Expression Omnibus (GEO ID: GSE50001).

2.6. Microarray data analysis and bioinformatics

Fold change in gene expression was determined from the non-log-transformed signal data after correction and normalization by using Ingenuity® iReport™. Gene expression fold change >1.5 or <−1.5 and $p < 0.05$ was set and considered significantly different from that observed in the control. Simultaneously, pathway analysis was performed, and the details can be found at the company website [<http://www.ingenuity.com/products/ireport> (ID: 2794)]. We also used the NCBI Database for Annotation, Visualization and Integrated Discovery (DAVID 6.7, <http://david.abcc.ncifcrf.gov>) for gene ontology analysis. A modified Fisher's exact test [35] was used to measure gene set enrichment. P -values <0.05 and an enrichment factor >2 were considered significant.

2.7. Quantitative real-time PCR (qRT-PCR) analysis

qRT-PCR was performed by using a RevertAid First Strand cDNA Synthesis Kit (Fermentas China Co. Ltd, Shenzhen, China) and a

GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) in a Mx3005P real-time QPCR system (Stratagene, La Jolla, CA, USA), following the manufacturer's protocols. The primers we used are listed in Table 1. Data quality control and analyses were performed according to the method described by Pfaffl and coworkers [36], and detailed information can be found in the Supplementary Material. Examination of the expression level of the housekeeping gene, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), in the microarray data showed no significant change between the control and treatment samples. Therefore, *GAPDH* was used as endogenous control.

2.8. Western blot analysis

The spleens were lysed with RIPA lysis buffer, and samples were separated by SDS-PAGE with 10% (15% for CD3G protein) separation gel for protein detection. The samples were transferred onto PVDF membrane (0.45 μ m), and the membranes were then blocked with 5% non-fat milk and washed in TBST (containing 0.1% Tween-20). The membranes were incubated with primary antibodies (1:1000 dilution) at 4 °C overnight, and after TBST washing, the membranes were incubated with the corresponding HRP-conjugated secondary antibody (1:5000 dilutions) for 1 h at room temperature. The membrane was further developed with the ECL Plus Western blotting detection system (Millipore Corporation, Billerica, MA, USA). The relative abundance of each protein was measured by using the Gel-Pro Analyzer software (Version 4.0, Media Cybernetics, Inc., Bethesda, MD, USA). β -actin was used as internal control.

2.9. Measurement of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)

The single-cell suspension of splenocytes was grown on 96-well plates at a cell density of 2×10^5 nucleated cells/mL for overnight (16 h) and then loaded with Fura-2/AM (5 μ M) for 45 min in cRPMI-1640 at 37 °C prior to PFOS exposure. The increase in intracellular $[Ca^{2+}]_i$ was expressed as the fluorescence intensity ratio measured at 340 nm and 380 nm excitation wavelengths (F340/F380), as it is proportional to calcium ion concentrations. F340/F380 ratiometric measurement of emission at 510 nm was obtained after 5 h and 24 h PFOS exposure. The

Table 1
Comparison of the gene expression ratios (T/C) as determined by microarray and quantitative RT-PCR analyses. Results were generated from triplicates ($n = 3$).

Gene symbol	GenBank accession no.	RT-PCR ^a	Array ^b	Sequence of primers used in RT-PCR
CACNA1A	NM_007578.3	1.54	1.7	F: 5'-tcctgtgctcgtttctgatg-3' R: 5'-acacgcacgtattcatccaa-3'
CAMK4	NM_009793.3	1.9	1.7	F: 5'-agcacattcaaacaccaca-3' R: 5'-ggactcagagatccgtctgc-3'
CD3G	NM_009850.2	1.71	1.5	F: 5'-atatctcattcgaggacagg-3' R: 5'-ccctgagtcctgctgagttc-3'
Themis	NM_178666.5	1.13	1.6	F: 5'-caccgaagacacaacaatgg-3' R: 5'-acagcattcattgccaaca-3'
MAPK12	NM_013871.3	4.11	1.6	F: 5'-gagacttgaagcctggcaac-3' R: 5'-ccgggttaccacatactcctg-3'
MAP3K5	NM_008580.4	1.3	1.5	F: 5'-aagaggcttgctggcataaa-3' R: 5'-tgctgtggtctctctagtt-3'
E2F1	NM_009692.3	0.15	0.5	F: 5'-agcctaggagattcagggtgt-3' R: 5'-cagagggtatggatcgtgct-3'
CDC25A	NM_007658	0.75	0.67	F: 5'-gggaagcatcaggatttgaa-3' R: 5'-cacccttgatgtgacctct-3'
CDK1	NM_007659.3	0.14	0.37	F: 5'-agctctgggcactcctaaca-3' R: 5'-ccattttgacagagattcgt-3'
CDKN1A	NM_007669	0.8	0.65	F: 5'-cacagctcagtgagctgaa-3' R: 5'-accctagaccacaatgcag-3'

^a Gene expression ratios determined by quantitative RT-PCR.

^b Gene expression ratios as determined by microarray analyses. F: Forward primer; R: Reverse primer.

experiment was conducted in the dark.

2.10. Statistical analyses

Data were expressed as the mean $\pm$ SD, and the student's *t*-test was used to compare differences between treatment and control groups, with the significance level set at $p < 0.05$.

3. Results

3.1. General toxicity and lymphoproliferation assay

During the exposure week (week 1–3), mice in all groups gained weight steadily over time and no significant difference in body weight was observed among treatment groups and control group (Fig. 1A). But in the recovery period (week 4), the body weight in 10 mg/kg/day group was lower than that of control. Spleen index also decreased in a dose-dependent manner during the recovery period (Fig. 1B). Histopathological examination showed that PFOS exposure resulted in alterations in spleen architecture (Fig. S2).

In general, Con A-induced T-cell proliferation was not affected during week 1–3, except for the first week in the 10-mg/kg/day

group, which caused significant ($p < 0.05$) inhibition of T-cell proliferation. Apparent dose-dependent inhibition of T-cell proliferation was observed during the recovery period (week 4), with the ratio decreasing from 1 to 0.85 and 0.84 in 5 mg/kg/day and 10 mg/kg/day groups ($p < 0.05$), respectively (Fig. 1C).

3.2. Lymphocyte phenotyping

At weeks 2 and 3, the percentage of CD3⁺ cells increased from 33.8% (control, week 2) to 48.7% and 40.7% (control, week 3) to 50.17% in the 5 mg/kg/day groups, respectively (Fig. 1D). After recovery, the abundance of CD3⁺ cells significantly increased in the 10 mg/kg/day group, with the percentage increasing from 39.87% to 52.8%. At week 3, the abundance of CD3⁺CD8⁺ cells increased in a dose-dependent manner, with the percentage increasing from 8.9% (control) to 10.8% and 11.7%, in the 5 mg/kg/day and 10 mg/kg/day groups, respectively (Fig. 1E). A similar trend was observed in the CD3⁺CD4⁺ cells, with the percentage increasing from 18.4% (control) to 23.7%, 25.7%, respectively (Fig. 1F). Although the percentage increased, the absolute number of T cell subpopulations decreased in the 5 mg/kg/day and 10 mg/kg/day groups in a dose-dependent manner during the recovery period (Fig. 1)G, H, and I.

Fig. 1. The effects of PFOS on body weight (A), spleen index (B), T-cell proliferation (C), and spleen cell composition (D–I) were measured in PFOS-exposed mice. The percentages of CD3⁺ (D), CD3⁺CD4⁺ (E), and CD3⁺CD8⁺ (F) cells were determined by flow cytometer for each group at weeks 1, 2, 3, and 4. The absolute cell numbers of CD3⁺ (G), CD3⁺CD4⁺ (H), and CD3⁺CD8⁺ (I) were normalized against the control group at each week, using a sample size of $n = 4$. Error bars represent S.D. Asterisks indicate significant difference from that observed in the control, at a significance level of $*p < 0.05$.

3.3. Gene expression profile and bioinformatics analyses

To further explore the underlying mechanisms of the PFOS side-effects in the spleen, gene expression profiling was conducted using microarray analyses. Based on the criteria of significance, changes in the expression of 1327 genes (4% of all analyzed genes) in the 10-mg/kg/day group were observed. Among these, 239 (0.8%) were upregulated, and 1088 (3.2%) were downregulated. Using the DAVID online tool, we further examined the functional distribution and category enrichment of the 1327 differentially expressed genes (DEGs) based on gene ontology terms. Of the gene list, 924 (69.6%) genes were mapped to the biological processes of the DAVID knowledge base. Sixteen biological processes were enriched (Fig. 2A), with the majority related to cell cycle/cell cycle control (15.7%, 145/924), DNA metabolism (7.5%), mitosis (5.8%), and DNA replication (4.3%), with respect to the whole mouse genome reference dataset. The enrichment analysis showed that in addition to DNA metabolism (enrichment factor, 3.7), replication (5.6), recombination (4.9), and chromosome segregation (3.9), a set of processes including porphyrin metabolism (6.8), MHCII-mediated immunity (3.7), hematopoiesis (3.2) and lipid and fatty acid transport (2.1) were also enriched, suggesting that these cellular processes were affected by PFOS. GO analysis showed that 15 molecular functions were significantly enriched, including alipoprotein (enrichment factor, 7.9), replication origin-binding protein (10.6), DNA-directed DNA polymerase (6.6), DNA helicase (4.4), and

kinase activator (2.6) (Fig. 2B).

We then used Ingenuity tools to perform pathway analysis of the DEGs to gather all the information on the molecular mechanism of PFOS-induced immunotoxicity. A total of 501 genes were mapped in the Ingenuity knowledge base, and based on criteria ($p < 0.05$ and >5 genes in the pathways), 66 pathways were significantly enriched in the treatment group. The full list can be found in [Supplementary Table S1](#). A few pathways were enriched because the same DEGs populated multiple pathways.

Examination of changes in gene expression patterns of these pathways showed that cell cycle-related pathways (including Cell Cycle Control of Chromosomal Replication, Role of CHK Proteins in Cell Cycle Checkpoint Control, Cell Cycle G2M DNA Damage Checkpoint Regulation, Cyclins and Cell Cycle Regulation, and Cell Cycle G1S Checkpoint Regulation) were most significantly downregulated by PFOS treatment (Fig. 3A). The pathways related to T-cell receptor (TCR) and signaling (primary immunodeficiency signaling, iCOS-iCOSL signaling in T helper cells, calcium-induced T lymphocyte apoptosis, and OX40 signaling pathway) were significantly upregulated, along with the downregulation of the anti-proliferative role of TOB in the T cell signaling pathway (Fig. 3B). The p38 MAPK signaling pathway represented a more complicated pattern of change in expression, with MAPK12 and MAP3K5 upregulated, whereas MAP2k3 was downregulated (Fig. 3C). In addition, gene (such as *GSTM3* and *MGST3*) expression in NRF2-mediated oxidative stress response decreased (Fig. 3D). The

Fig. 2. Function analysis and category enrichment analysis of differentially expressed genes (DEGs). (A) Biological processes; (B) Molecular function. Number of DEGs: the number of differentially expression genes belongs to one category in the genome database; Expected number of genes: the number of genes having an ontology annotation in the reference database.

Fig. 3. Microarray analysis of the expression levels of differentially expressed genes (DEGs) in mouse spleens with (10 mg/kg/day) or without (control) PFOS exposure. DEGs are involved in (A) cell cycle control related pathways, (B) T-cell receptor (TCR) and signaling, (C) p38 MAPK signaling, and (D) NRF2-mediated oxidative stress response.

corresponding information on these pathways is presented in Table S2. These results showed that PFOS had an important effect on these pathways. Among them, the ones related to T-cell development and signaling are of particular biological significance in terms of the immunotoxicity of PFOS because T cells play an important role in cell-mediated immunity.

To further confirm the results of microarray analyses, we selected a few key genes involved in these pathways and used QRT-PCR to quantify changes in levels of expression. These genes were determined to be related to: TCR signaling (*CD3G* and *Themis*), calcium ion regulation and signaling, including *CAMK4* and *CACNA1A*, MAPK signaling (*MAPK12* and *MAP3K5*), and cell cycle regulation (*E2F1*, *CDC25A*, *CDK1*, and *CDKN1A*). A similar trend in was observed between QRT-PCR and the microarray data (Table 1).

3.4. Western blot assay and intracellular free Ca^{2+} concentration

It is well known that Ca^{2+} plays an important role in the proliferation of immune cells in response to foreign antigens [37]. Therefore, interference of Ca^{2+} might be involved in the immunotoxicity of PFOS. Based on above results, we measured the protein expression levels of *Themis*, *CD3G*, and *CAMK4*, due to the fact that they are closely related to TCR signaling and calcium signaling. In general, the change in the expression of these proteins was more prominent in week 4 than in week 3, and was dose-dependent. For example, the expression level of *Themis* increased by 1.2- and 1.5-fold at weeks 3 and 4 in the 10 mg/kg/day group, respectively. *CAMK4* was significantly ($p < 0.05$) upregulated at week 4 in the 5 (2.4-fold) and 10 (2.5-fold) mg/kg/day groups. Similarly, the level of *CD3G* expression changed by 0.9- and 1.9-fold in the 5 and 10 mg/kg/day groups, respectively, at week 3. A more prominent dose-response was observed at week 4, with the expression levels increasing by 1.5- and 2.5-fold, respectively (Fig. 4).

In addition, based on microarray analysis, which shows that

PFOS could modulate Ca^{2+} -induced T cell apoptosis pathway, we further measured the $[\text{Ca}^{2+}]_i$ of splenocytes after acute PFOS exposure to illustrate the effects of PFOS on calcium homeostasis. A series of acute PFOS exposure at concentrations $>25 \mu\text{M}$ (approximately $12.5 \mu\text{g/mL}$, the highest occupational plasma level) was used to simulate a low concentration long-term effect. Cell viability was not affected by these treatments for as long as 24 h (Fig. S3). As shown in Fig. 5, Con A (positive control) induced an increase in $[\text{Ca}^{2+}]_i$ by 1.45-fold. After 5 h of PFOS exposure, $[\text{Ca}^{2+}]_i$ increased in a concentration-dependent manner, with the F340/F380 ratio increasing from 1.19 (control) to 1.23, 1.32, and 1.32 in 100, 200, and 500 μM treatment groups, respectively (Fig. 5A). A similar trend was observed after 24 h exposure, with higher ratios (Fig. 5B).

4. Discussion

The PFOS concentration in the general population and in nature was at the ng/mL level. Recently, an investigation on dust around fluorochemical plant revealed extremely high PFOS concentrations, ranging from 2.68 to 18,486 ng/g (or 18.486 mg/kg) [38], indicating that the occupational situation apparently caused much higher exposure levels of PFOS to workers, the resulting serum concentration could reach as high as $>10 \mu\text{g/mL}$ level [3,11]. A study employing 5–40 mg/kg/day exposure treatment showed that PFOS could induce splenic atrophy and lymphocyte disorders [19]. Studies using a similar range of exposure dosage (5–20 mg/kg/day) for 7–14 days or a 10-day treatment with dietary doses (1%–0.001%, PFOS w/w) revealed the destructive effect of PFOS on splenic structure, changes in inflammatory response, and cytokine production [20,28,33]. Therefore, we used up to 2.5–10 mg/kg/day as the treatment to simulate the high occupational exposure scenario. Several previous studies based on similar exposure framework revealed that exposure of male C57BL/6 mouse to 50, 125, 140,

Fig. 4. Western-blot and gel image analyses (A) of the levels of protein expression of Themis (73 kDa) (B), CAMK4 (55 kDa) (C), and CD3G (20 kDa) (D) of mouse spleens exposed to PFOS (control, 5, and 10 mg/kg/day) for 3 and 4 weeks. Error bars represent S.D. from three individual experiments. The asterisk indicates significant difference from that of the control at a significance level of $*p < 0.05$.

Fig. 5. $[Ca^{2+}]_i$ in mouse splenocytes after 5 h (A) and 24 h (B) of PFOS exposure. Error bars represent S.D. Asterisks indicate significant differences from that observed in the control, at a significance level of * $p < 0.05$.

200 and 280 mg/kg TAD resulted in serum PFOS concentrations of 51.7–59.7 mg/L [21,24], 120.7 mg/L [16], 250 mg/L [20], 340 mg/L [39], and 338 mg/L [19], respectively. The TADs used in our experiment were 52.5 (lowest) and 210 mg/kg (highest) and therefore the lowest serum PFOS concentration would be around 50–60 mg/L and the highest concentration would be around 340 mg/L at the end of exposure, respectively. In the note of different mouse strains (BALB/c in this study vs. C57BL/6), the measurement should be included in our future works and more studies are needed to analyze the effects of long-term exposure to lower dosages of PFOS on immune system. In the present study, when mice were exposed to comparable dosages of PFOS (2.5–10 mg/kg/day), the results showed that although PFOS caused atrophy of the spleen and a decrease in the absolute number of T-cell subpopulations, the percentages of these cells ($CD3^+CD4^+$ and $CD3^+CD8^+$) increased. The result is consistent with the findings of a few studies that described interference of perfluorinated chemicals in T lymphocyte subsets by decreasing the absolute number of these cells in adult male mice [16,40]. In addition, inhibition of the T-cell response to a mitogen was suggestive of the anergy of this cell population after PFOS exposure.

During PFOS exposure (weeks 1–3), majority of the toxic effects on spleens were not significant until the recovery week (week 4), indicating that the spleens underwent a series of molecular events during the exposure, which led to a delayed type of toxicity to PFOS. Our research group is particularly interested in the alterations occurring right before the cessation of PFOS exposure. Therefore, we determined the global gene expression profiles of spleens in highest treatment group (10 mg/kg/day) at the time point of week 3. Comparison of the expression patterns of the control and treatment groups showed that a variety of cell cycle-related pathways, including cell cycle control of chromosomal replication, G1/S checkpoint regulation, G2/M DNA damage checkpoint regulation, CHK proteins in cell cycle checkpoint control, and cyclins and cell cycle regulation were inhibited (Fig. 3A). Similar results were previously reported in PFOS-exposed bottlenose dolphin skin cells and splenocytes, thus indicating regulation of cell cycle progression and cell proliferation using stage-specific checkpoints [21,41]. The main mechanism of the cell cycle checkpoints involves the regulation of the cyclin-dependent kinase (CDK) activities by CDC25A [42]. CDKs are protein kinases that modulate the activity of its substrates, which include the transcription factors E2F (E2F1) and its regulator Rb, thereby controlling cell cycle progression. The decrease in CDK1 activity results in a delay in cell cycle progression in human T lymphocytes, which in turn leads to the impairment of the proliferative response of T lymphocytes to mitogenic stimulation [43].

Therefore, the downregulation of *CDC25A*, *CDK1*, and *E2F1* by PFOS exposure could inhibit cell cycle progression, resulting in the decrease in the rate of T lymphocyte proliferation upon Con A stimulation, as shown in Fig. 1C. The massive deregulation of cell cycle checkpoint pathways could also contribute to the decrease in the number of T cells in the spleen (Fig. 1)H and I.

More importantly, several pathways directly related to the generation of immune cell responses were identified to be modulated by PFOS, including primary immunodeficiency signaling, iCOS-iCOSL signaling in helper T cells, anti-proliferative role of TOB in T cell signaling, OX40 signaling pathway, and calcium-induced T lymphocyte apoptosis. These pathways converge at the MHC-II- and TCR-mediated immunity and calcium signaling cascade as a group of genes that involve calcium regulation (such as *CAMK4* and *CAMK2d*), and the MHC-II complex (i.e., *H2-T24*, *H2-K1*, *H2-DMB1*, *H2-T10*, *H2-Ab1*, and *H2-oA*) and TCR (i.e., *CD3G*, *CD3d*, and *LCK*) were present in multiple pathways and were significantly upregulated at the transcriptional or protein levels (Figs. 3B and 4). Calcium ions are involved in various important biological processes, and calcium-mediated signaling pathways play crucial roles in a wide range of cellular events, including cell proliferation and differentiation, apoptosis, and autophagy [44]. The effects of PFOS on calcium channel downstream signaling have been reported in the nervous system, revealing a molecular mechanism of its neurotoxicity [44]. In the present study, calcium signaling in lymphocytes was determined to be modulated by PFOS, and an increase in intracellular Ca^{2+} concentration was detected after exposure to this particular reagent (Fig. 5). Prolonged and relatively higher Ca^{2+} concentrations could activate a sequence of downstream signaling pathways and disrupt the interactions between T-cells and antigen-presenting cells in inducing alterations in cytoskeletal organization and motility [45], developmental arrest, unresponsiveness, and apoptosis of T lymphocytes [46,47]. Therefore, the calcium signaling pathway and disturbances in free intracellular Ca^{2+} concentrations might be involved in the mechanism of PFOS immunotoxicity.

Themis is expressed in spleen, regulates calcium influx in cells, and activate TCR signaling during T-cell maturation. Changes in the expression of *Themis* occur during developmental immaturity [48]. Therefore, the PFOS-induced upregulation of *Themis* suggests its disruptive effects on the maturation of T-cells, which eventually leads to T-cell anergy. Similarly, *CD3G*, along with *LCK*, can form a complex with TCRs. Defects in these genes are associated with T cell immunodeficiency and dysfunction of mature T-cells [49,50]. The order and ratio of CD3 peptides (i.e., gamma, epsilon, and delta) in association with TCRs are tightly regulated during the maturation of

T cells, and its disruption can lead to biased T cell proliferation [51]. CAMK4 operates in the calcium-triggered CaMKK-CaMK4 signaling cascade and regulates the activity of transcription activators, by which CAMK4 links T-cell antigen receptor (TCR) signaling to the production of IL-2, IFN- γ , and IL-4 [52–54], which in turn determines the nature of an immune response. Recently, PFOS was shown to suppress IL-2 production in both human T cell lines and primary T-cells [55], indicating that the disruption of TCR signaling by PFOS exposure might cause the arrest of T-cell proliferation and altered immune responses. Interestingly, Negoro and coworkers [56] showed that the incidence of asthma was caused by an increase in $[Ca^{2+}]_i$ of regulatory T-cells following TCR activation, with partial loss of regulatory functions, indicating that PFOS-induced calcium influx and TCR activation might be responsible for juvenile asthma, as reported by Dong et al. [14].

In addition, CAMK4 can activate MAPK14/p38 and stimulate transcription of downstream genes [53,57]. MAPK pathways play a crucial role in both adaptive and innate immunities, regulating immune responses [58,59]. Interestingly, our microarray data showed that the p38/MAPK pathway was modulated by PFOS, with the upregulation of stress-activated MAP kinase genes (such as *MAPK12* and *MAP3K5* in Table 1 and Fig. 3B). It has been reported that PFOS-activated p38/MAPK pathways could induce apoptosis of cerebellar granule cells and zebrafish embryo cells [60,61]. Therefore, the activation of MAPK pathway by PFOS may result in immune cell apoptosis and thus atrophy of the spleen. Alternatively, the pathways can also be activated by oxidative stress [62]. It has been illustrated that PFOS could overwhelm the homeostasis of anti-oxidative systems in orally PFOS-exposed adult male C57BL/6 mice, resulting in ROS overproduction and decreased glutathione-S-transferase (GST), as well as splenocyte apoptosis [22]. The results of our microarray analyses showed that the NRF2-mediated oxidative stress response pathway was inhibited by PFOS exposure, along with the downregulation of *GSTM3* and *microsomal GST* (*MGST3*), as shown in Fig. 3D, suggesting that the role of oxidative stress in induction of MAPK pathways might not be excluded.

Future studies should investigate the gene expression profiles of the thymus because T cells originate from the bone marrow and mature in the thymus, which are then released to the peripheral tissues. In addition, the thymus has been shown to accumulate PFOS at a similar magnitude as that observed in the mouse spleen [17]. In addition, the effects of PFOS on the thymus have been previously reported [16]. The results of such work could provide more clues on the potential toxic mechanisms of PFOS. Furthermore, the percentage of T cells generally increased, although the absolute number of T cells decreased in a dose-dependent manner, indicating a severe decrease in other cell populations in the spleen. Therefore, future work should focus on other immune cells using various dosage and time points.

5. Conclusions

In summary, the ubiquitous PFOS contamination poses a potential threat to the environment and human health, and its immunotoxicity raises concern particularly when its molecular mechanisms have not been fully established. The present study examined the toxic effects and the underlying mechanisms of PFOS in the BALB/c mouse spleen. The results showed that PFOS induced spleen atrophy, as well as changes in spleen architecture and composition of T-cell subpopulations. T-cell proliferation in response to Con A stimulation was inhibited by PFOS exposure. Most of these effects were not prominent during the exposure period of PFOS until after a week recovery. Microarray analyses showed that during the PFOS exposure, a variety of cell cycle regulation pathways were inhibited, along with the activation of

pathways related to T-cell signaling and development and calcium ion influx. The altered signaling pathways by PFOS treatment as revealed by the present study might facilitate in better understanding PFOS immunotoxicity and shed light on the toxic mechanisms and pathogenesis of PFOS exposure.

Conflict of interest statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.cbi.2015.07.015>.

Transparency document

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